ENGINEERING THREE DIMENSIONAL CULTURE PLATFORMS FOR HUMAN ADIPOSE DERIVED STEM CELL THERAPY

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ANJANEYULU KODALI

(M.Tech., INDIAN INSTITUTE OF TECHNOLOGY (BANARAS HINDU UNIVERSITY), INDIA)

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Anjaneyulu Kodali

Date: 6 January 2014

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CONTENTS

SUMMARY

The overall objective of this work is to devise a tissue engineering strategy to enhance the therapeutic potential of human adipose derived stem cells (ADSCs) using three dimensional microsphere (3D) scaffolds and to fabricate such cell-scaffold constructs into a suitable delivery system for clinical applications. To achieve this objective, we initially employed 3D gelatin microspheres (GMs) to form compact cell-microsphere constructs (ADSC-GMs) with ADSCs and investigated the tissue regenerative properties of those constructs. We hypothesized that ADSC-GMs with their strong cell-cell and cell-matrix interactions will aid in improving the biological functional abilities of ADSCs. Later, to make these constructs feasible for *in vivo* delivery, we encapsulated them into *in situ* gelling collagen hydrogels to form hydrogel-microsphere composite scaffolds (Col-GMs).

To begin with, ADSC-GM constructs were formed by culturing ADSCs on the 3D surfaces of the microspheres and the role of GMs in controlling various properties of ADSCs was studied. We studied their proliferation, maintenance of stemness, differentiation into various lineages and finally their pro-angiogenic properties. All these properties play a key role in tissue regeneration and enhancing such properties will be beneficial for tissue regeneration. Firstly, we studied the stemness properties of ADSC-GMs by conducting gene expression studies for the four well known pluripotent markers genes Oct4, Sox2, Nanog and Rex1. We found that all these genes were significantly upregulated in ADSC-GMs while in the ADSCs cultured on two dimensional (2D) tissue culture dishes, except Rex1 all other genes were found to be down regulated. Then we studied the differentiation abilities of ADSC-GMs into three different lineages, namely – adipogenic, osteogenic and hepatic lineages. Our results show that ADSCs cultured on GMs were able to successfully differentiate into all the three lineages showing enhanced expression of respective marker genes compared to 2D

cultures. Finally, using the *in vitro* HUVEC-matrigel assay, we demonstrated that ADSC-GMs have enhanced pro-angiogenic properties compared to ADSCs cultured on 2D. This would lead to better vascularisation of the regenerating tissue. In conclusion, this part of our work shows that ADSC-GM constructs have enhanced regenerative properties compared to conventional 2D cultures. Employing these constructs for treating damaged tissues would accelerate tissue regeneration and hence, enhances the therapeutic potential of ADSCs for tissue regenerative applications.

The second part of this thesis focuses on making these constructs with enhanced regenerative properties feasible for *in vivo* delivery, for an easier transition of these systems into a clinical setting. To this end, we formed composite hydrogel scaffolds (Col-GMs) by encapsulating the ADSC-GMs into injectable, *in situ* gelling collagen hydrogels. Incorporation of GMs into collagen hydrogels varies the mechanical properties of the hydrogels and hence allows for tuning the rigidity of the hydrogels to provide appropriate mechanical cues for the encapsulated cells. In addition, the encapsulated GMs can be used as depots for growth factors and can in turn provide with the required biomolecular cues. Thus, in this system of Col-GMs, we further studied the effect of mechanical and biomolecular cues provided by the scaffolds on the osteogenic differentiation of the ADSCs. We found that incorporation of GMs into the collagen hydrogels enhances the storage modulus of the hydrogels and further favours osteogenic differentiation of the encapsulated ADSCs. Presentation of biomolecular cues such as controlled release of basic fibroblast growth factor (bFGF) from the GMs also seems to have a promoting effect on the osteogenic differentiation of ADSCs compared to bFGF supplementation in the medium. Overall, this part of our study shows that Col-GM composite scaffolds can regulate the osteogenic differentiation ability of ADSCs and can potentially be used as effective injectable delivery vehicles for ADSC-GMs with the ability to control release growth factors.

In conclusion, the work presented in this thesis shows that, 3D GMs can aid in enhancing the regenerative properties of the ADSCs along with having the potential to take part in the vascularisation of regenerating tissues. Further, we

also showed that, osteogenic induction of ADSCs can be enhanced through presentation of appropriate mechanical and biomolecular cues in the Col-GM composite scaffolds which can in turn be used as delivery vehicles for ADSC-GMs. Overall, both ADSC-GMs and Col-GM strategies presented in this thesis, can be promising approaches for stem cell culture and delivery and can be employed for stem cell based regenerative therapies.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ADSCs	Adipose derived stem cells
ADSC-GMs	Cell-microsphere constructs formed using adipose derived stem cell and gelatin microspheres
AFP	Alpha-fetoprotein
ALB	Albumin
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
bFGF	basic fibroblast growth factor
BMP2	Bone morphogenetic protein 2
BMSCs	Bone marrow mesenchymal stem cells
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CLSM	Confocal laser scanning microscope
Col	Collagen hydrogel

Col-GMs	Collagen hydrogel – gelatin microsphere composite scaffolds
Col-10-GMs	Collagen hydrogel containing 10 mg of gelatin microspheres
Col-20-GMs	Collagen hydrogel containing 20 mg of gelatin microspheres
Cyt18	Cytokeratin 18
DI	Deionized
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extra cellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GMs	Gelatin microspheres
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cells
iPSCs	induced pluripotent stem cells
MSCs	Mesenchymal stem cells
OCN	Osteocalcin

PBS	Phosphate buffered saline
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
SEM	Scanning electron microscope
TRITC	Tetramethylrhodamine B isothiocyanate
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

A brief background, motivation, hypothesis and objectives of this thesis work will be presented in this chapter.

1.1. Background and motivation

Stem cell therapies are gaining increased popularity over the last decade or so, because of the advent of a variety of adult stem cells and the kind of impact that such therapies can create on the status quo medical treatments. Adult stem cells, unlike embryonic stem cells, are not embroiled with ethical issues and can be used in autologous fashion. They can also be easily differentiated to various specific cell types and do not form teratomas *in vivo*. With all these advantages, adult stem cells seems to be a potential alternative to embryonic stem cells and also opens up a new avenue with immense therapeutic value for treating organ failures.

Adipose derived stem cells (ADSCs) which are present in adipose tissue are one such kind of adult stem cells. They are categorized as mesenchymal stem cells (MSCs) and have very similar characteristics to that of bone marrow derived mesenchymal stem cells (BMSCs) (Kern et al. 2006). ADSCs have a lot of advantages compared to other types of adult stem cells, such as availability in large numbers, ease of harvesting the fat tissue and their multi-lineage differentiation ability (Parker et al. 2006). Typically $5x10^7 – 6x10^8$ ADSCs can be obtained by processing 300 mL of lipoaspirate with very high cell viabilities of greater than 90% (Zuk et al. 2001, Aust et al. 2004). All these advantages make them an ideal choice of cell source for stem cell regenerative therapies.

Although stem cell therapies seem to be very attractive, their feasibility of becoming a viable medical treatment strategy hinges on being able to overcome a few challenges. Firstly, there is a need to develop suitable platforms which can support stem cell propagation with proper maintenance of their stemness properties and also support their multi-lineage differentiation ability. Secondly, to design strategies that makes such *in vitro* culture platforms suitable for *in vivo* delivery applications by minimally invasive means.

In this thesis, we aim to address mainly these two challenges. We have employed three-dimensional (3D) gelatin microspheres (GMs) as cell culture platforms and investigated their viability for tissue engineering with ADSCs. To this end, we formed cell-microsphere constructs (ADSC-GMs), by culturing ADSCs on GMs and further studied some of their properties that play crucial role in tissue regeneration – proliferation, stemness maintenance, multi-lineage differentiation and pro-angiogenic properties.

Subsequently, to make the ADSC-GMs more suitable for *in vivo* delivery, we encapsulated them into collagen hydrogels which can gel *in situ* and can be delivered by injectable means. The hydrogel-microsphere composite scaffolds (Col-GMs) thus formed have the capability to provide both mechanical and biomolecular cues to the encapsulated ADSCs. Appropriate mechanical cues can be provided by varying the amount of encapsulated GMs which changes the rigidity of the scaffold. On the other hand, the GMs can also be used to control release required growth factors and in turn can help in providing the appropriate biomolecular cues. Thus in Col-GMs, we also investigated the effect of such mechanical and biomolecular cues on the osteogenic differentiation of ADSCs.

Overall, we believe that the hydrogel-microsphere composite system that we developed in this work can be effectively used as an injectable stem cell delivery strategy for adipose derived stem cell therapy.

1.2. Hypothesis

The three dimensional cell-microsphere (ADSC-GMs) constructs formed using ADSCs and GMs with strong cell-cell and cell-matrix interactions can enhance the tissue regenerative properties of human ADSCs compared to traditional two dimensional tissue culture plates. Also, it is hypothesized that the behaviour of such ADSC-GM constructs can be modulated by encapsulating them in collagen hydrogels and providing with appropriate mechanical and biomolecular cues.

1.3. Objectives

To investigate the above given hypothesis, following objectives were laid down.

1) To fabricate ADSC-GM constructs and study the effect of GMs on the tissue regenerative properties such as proliferation, stemness maintenance, multi-lineage differentiation and pro-angiogenic properties of human ADSCs (Chapter 4)
2) To fabricate and characterize hydrogel-microsphere (Col-GMs) composite scaffolds by incorporating GMs in collagen hydrogels with varying mechanical and biomolecular cues (Chapter 5).
    a) Fabricate Col-GMs by encapsulating different amounts of GMs in collagen hydrogels and study their mechanical properties by performing rheological studies.
    b) Encapsulate basic fibroblast growth factor (bFGF) into Col-GM scaffolds and study the release profiles *in vitro* using ELISA.
3) To study the effect of mechanical and biomolecular cues provided by the Col-GM scaffolds on ADSC behaviour (Chapter 5).
    a) Investigate the effect of Col-GMs mechanical properties on ADSCs by differentiating them towards osteogenic lineage
    b) Investigate the effect of bFGF controlled release on the osteogenic differentiation of ADSCs.

CHAPTER 2

LITERATURE REVIEW

A description of stem cell based tissue regenerative approaches with a focus on biomaterials/injectable scaffolds employed for stem cell therapies will be provided in this chapter.

2.1. Tissue engineering and regenerative medicine

Human organs can get damaged due to various reasons such as diseases or accidents. But the only medical treatment approach that is currently under practise is organ transplantation. Although surgeons world over have been employing this method for a few decades, it is still associated with some severe drawbacks, mainly donor organ shortage and immune rejections. To overcome these problems, a completely new approach to treat organ failures was put forward by a group of clinicians and material scientists which was popularly termed as tissue engineering (Langer et al. 1993). The overall objective of tissue engineering as coined at the emergence of this field is to fabricate fully functional off the shelf tissues which can act as biological substitutes for damaged tissues. Although this goal seems to be a few decades away, few significant milestones have already been reached, such as generation of induced pluripotent stem cells (iPSCs) (Takahashi et al. 2006), isolation of stem cells from adipose (Zuk et al. 2001) and other adult organs (Korbling et al. 2003), direct reprogramming of fibroblasts to heart (Ieda et al. 2010) and neural cells (Vierbuchen et al. 2010), implantation of a tissue engineered airway into a human patient (Macchiarini et al.) and controlled design of various scaffolds using biomaterials (Hollister 2005). In slight contrast to tissue engineering, regenerative medicine approaches mainly focus on cell therapies using suitable delivery vehicles which can support *in vivo* tissue regeneration upon implantation. Various kinds of stem cells are being studied for their suitability to such cell therapies which will be discussed in the following sections. Over the last decade, tissue regenerative approaches are gaining more popularity compared to the highly ambitious tissue engineering motto of “selling artificial organs”. Another major area of focus in regenerative medicine has been the development of biomaterials which can act as injectable delivery vehicles for such cell therapies as well as controlled release biomolecules in a spatio-temporal manner, which will also be discussed in the subsequent sections of this chapter.

The growing interest in the potential of this field is also evident from the increase in the number of registered clinical trials in the US which are underway. The clinical trials in the field of tissue engineering and regenerative medicine have

risen from 38 in 2007 to 83 in 2011 (Fisher et al. 2013). The outcomes of these trials will further aid us in assessing the true potential of various approaches that are being employed and helps us in taking corrective actions to further improve those approaches for clinical applications.

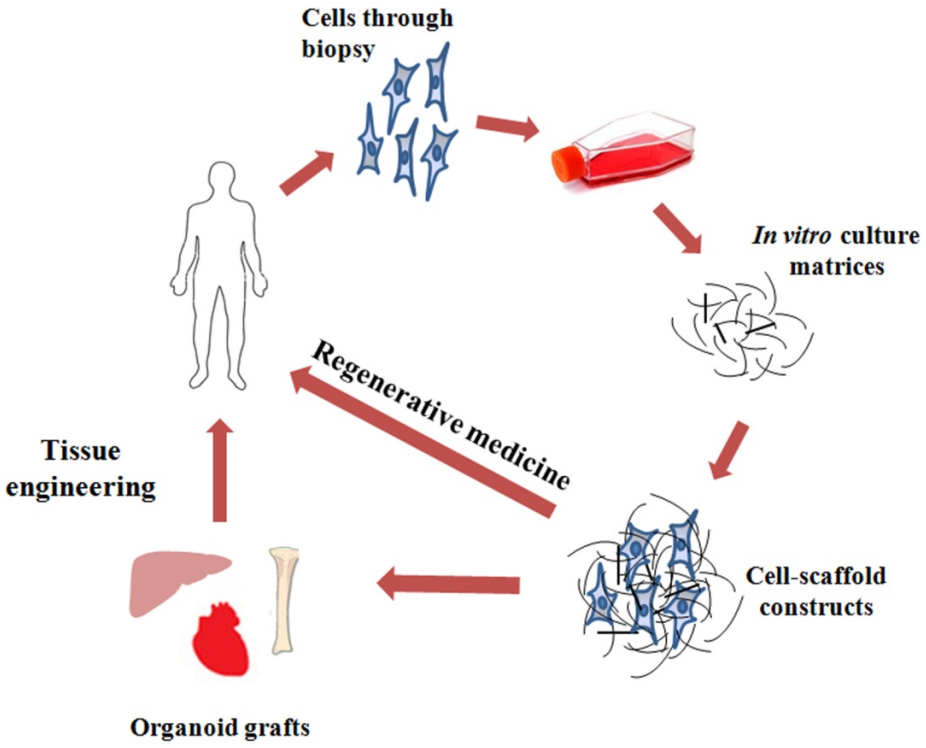

Figure 2.1 Schematic showing a general sequence of steps involved in tissue engineering and regenerative medicine strategies. Cells are isolated from the donor tissue sections obtained through biopsies which are expanded *in vitro* and seeded on 3D cell culture matrices made of biomaterials to form cell-scaffold constructs. In regenerative medicine approach, either aqueous cell suspensions or cell-scaffold constructs are directly injected back into the patient to assist the natural process of tissue regeneration. On the other hand, in tissue engineering, such cell-scaffold constructs are then used to fabricate fully functional organoid grafts which will be implanted into the patients to regain the tissue functions.

2.2. Stem cells in tissue regeneration

Cell therapies are fundamental to most of the tissue regenerative approaches and finding a reliable source for the supply of cells has been a major area of focus. Cells can be harvested from autologous tissues which are partly injured but such procedures are associated with intense morbidity. Also, in many instances when the tissue is severely damaged, not many good quality cells can be harvested from those tissues. Advancements in the field of stem cell biology have opened up new options of stem cell based tissue regenerative therapies. As stem cells can be induced to differentiate into multiple cell types, the differentiated cells obtained can then be used as replacements for the damaged cells within a specific tissue. This led to further investigations about the suitability of various types of stem cells for such stem cell based therapies, few of which are discussed in the following sections. Stem cells are broadly classified into embryonic and adult stem cells based on their origin.

2.2.1. Embryonic stem cells

Cells with pluripotent nature were isolated from the inner cell mass of the mouse embryos and thus were termed as embryonic stem cells (Martin 1981). Later, these cells were also isolated from inner cell mass of human blastocysts (Thomson et al. 1998) which started all the controversy surrounding ESCs that is existent even today. These cells are an ideal source for tissue engineering applications as they can self-renew indefinitely and can differentiate into cell types of all the three germ layers. However, there are major drawbacks associated with these cells such as the ethical issues, teratoma formation upon *in vivo* implantation and their allogenic source which invokes immune response. These drawbacks limit their wider usage for clinical applications.

2.2.2. Adult stem cells

Stem cells that regularly take part in the replenishment of dead cells and in the regeneration of damaged tissues have been found to be present in many tissues of the adult body. Depending on the type of stem cell, their differentiating capacity and their potency will vary. Some cells can differentiate into only one specific lineage and are termed as progenitor cells. Other stem cells from some tissues are multipotent and can give rise to cells that are not related to their source tissue. For example, bone marrow (Pittenger et al. 1999) and adipose (Zuk et al. 2002) tissues are two widely popular sources for mesenchymal stem cells which can give rise to a wide variety of cell types. Many adult stem cells are proving to be promising alternatives for ESCs because of their similar differentiation abilities, ease of availability and being able to be used in autologous fashion. However, harvesting cells from adult tissues obtained through biopsies involves some problems such as morbidity and low cell numbers. Thus for clinical applications, it will be advantageous to find ways to harvest tissues by minimally invasive means which contain large numbers of stem cells.

2.2.3. Adipose derived stem cells

ADSCs are adult stem cells found in adipose tissues with very similar characteristics to BMSCs. They were first isolated in 2001 (Zuk et al. 2001) and since then they were gaining increased popularity over other adult stem cells because of many advantages. Adipose tissues can be harvested by minimally invasive means such as liposuction with local anaesthesia. This makes ADSCs to be easily available compared to other stem cells and can be used in autologous fashion. They are also available in very high densities in fat tissues with typical cell numbers of around $5x10^7$ – $6x10^8$ from 300 ml of lipoaspirate (Zuk et al. 2001, Aust et al. 2004) which is approximately 40 times higher compared to BMSCs (Strem et al. 2005). In addition, ADSCs also seem to have higher immunomodulatory capacity compared to BMSCs (Melief et al. 2013). They also exhibit high proliferation rates along with multi-lineage differentiation ability (Zuk et al. 2002). With all these advantages, ADSCs are proving to be a promising cell source for tissue regenerative applications and are being widely investigated both at lab scale and also at clinical scale (Gir et al. 2012).

ADSCs have been shown to be able to differentiate into various lineages including adipogenic, osteogenic, chondrogenic, myogenic, neural and hepatic cells (Talens-Visconti et al. 2007, Bunnell et al. 2008a, Cardozo et al. 2012, Sung et al. 2013). While the differentiation into former three lineages has been widely known and has well established protocols, differentiation into the later three lineages is more challenging and is currently under study by various research groups. In this thesis, we attempted differentiating ADSCs into hepatic lineage along with adipogenic and osteogenic lineages on 3D gelatin microspheres with an objective of making use of such differentiated cell-microsphere constructs for liver, fat and bone tissue reconstruction.

2.2.4. Hepatic differentiation of ADSCs

Liver tissues have a unique ability to regenerate after an injury. Hepatocytes and liver progenitor cells are the main cells responsible for the regenerative feature of liver. In case of an acute injury, hepatocytes will first respond with high proliferating rates (Fausto et al. 2005). Liver progenitor cells will form a reserve pool of cells which will start to proliferate and differentiate in case of a failure in hepatocyte proliferation (Roskams et al. 2003). However, in case of end stage liver disease, most of liver cells gets damaged and the liver looses the ability to regenerate. In such cases, stem cell transplantation is being looked into as a potential treatment strategy. ADSCs are being studied for their ability to differentiate into hepatocytes because of their advantages over other stem cells as mentioned in previous section. In 2005, Seo et al. has first shown that, human ADSCs can be induced towards hepatic lineage using hepatocyte growth factor and oncostatin M as media supplements (Seo et al. 2005). Since then, there has been increased interest in the hepatic potential of ADSCs and different combinations of growth factors have been tried (Talens-Visconti et al. 2007, Yamamoto et al. 2008, Aurich et al. 2009, Coradeghini et al. 2010, Banas 2012). However, most of the studies were performed on 2D tissue culture dishes with very few in 3D scaffolds. For instance, Wang et al. has studied hepatogenesis of ADSCs in 3D PLGA scaffolds (Wang et al. 2010). From tissue engineering perspective, it is important to understand the hepatic potential of ADSCs in 3D

scaffolds and thus more such studies need to be performed in scaffolds made of various biomaterials. Such differentiated hepatocyte-scaffold constructs can in turn be used as implants for treating liver damages and also for conducting drug screening studies.

2.2.5 Characterization methods for adipogenic, osteogenic and hepatic differentiation of ADSCs

Characterization of stem cell differentiation is usually done by histochemical staining methods or by studying the expression of specific lineage marker genes or proteins. Various methods that are most commonly employed for characterizing adipogenic, osteogenic and hepatic differentiation of ADSCs is discussed below.

Adipogenic differentiation of ADSCs can be very easily identified under an optical microscope by the deposition of lipid bodies inside the differentiated ADSCs. Such lipid bodies can further be stained using Oil red O stain to have a distinct appearance. For further in depth characterization, gene expression of adipogenic marker genes such as peroxisome proliferative activated receptor γ (PPAR-γ), fatty acid-binding protein 4 (FABP4) and lipoprotein lipase can be studied using qPCR (Hu et al. 2011b). In addition, as glycerol-3-phosphate dehydrogenase (GPDH) activity is increased upon adipogenic differentiation, GPDH assay can also be employed for characterizing the extent of differentiation.

For characterizing osteogenic differentiation of ADSCs histochemical staining methods using alizarin red S and von kossa are usually employed to visualize the mineralized matrix deposition of differentiated ADSCs. Both alizarin red and von kossa can bind to the mineral depositions produced by the differentiated osteoblasts. Von Kossa method is based on binding of the silver ions to the anions such as phosphates of calcium salts and the reduction of silver salts to form dark brown or black metallic silver staining. On the other hand, alizarin red S reacts directly with calcium cation to form a chelate. Further characterization can be done by studying the genetic or protein level expression of osteogenic markers such as runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP),

bone morphogenetic protein 2 (BMP2), osteopontin (OPN) and osteocalcin (OCN) (Hu et al. 2011b).

Hepatic differentiation of ADSCs can be characterized using Periodic acid-schiff (PAS) staining to identify the glycogen storage ability of the hepatic differentiated ADSCs. In addition, gene or protein level expression of various hepatic markers such as albumin (ALB), alpha-fetoprotein (AFP), cytokeratin 18 (Cyt18), transthyretin (TTR) (Banas et al. 2007), can also be studied for characterizing hepatic differentiation.

2.3. Biomaterial scaffolds for Stem cell therapies

Traditionally, many of the studies involving stem cells have been performed on 2D tissue culture plates. Although such studies have provided us with most of the existing knowledge of stem cell biology, they have certain limitations. Firstly, these culture systems cannot fully replicate the *in vivo* milieu which mainly consists of extra cellular matrix (ECM) that provides a unique biological niche for the cells to adhere and differentiate. Secondly, such stem cells cultured on 2D, have also proven to be in efficient for tissue regeneration (Lee et al. 2008, Mooney et al. 2008). Further, direct injection of such cell suspensions has lead to the death of the transplanted cells in many instances (Guerette et al. 1997, Emgard et al. 2003). Shortage of cell-matrix interactions leading to anoikis was found to be the reason for such cell death (Terrovitis et al. 2010). On the contrary, stem cell delivery using 3D delivery vehicles which can support cell adhesion were able to improve the survival rate and their tissue regenerative ability (Zakharova et al. 2010, Parisi-Amon et al. 2013). This highlights the need for developing 3D delivery platforms which can mimic the ECM. The materials for developing such 3D platforms also have to be biocompatible as well as biodegradable which are in turn termed as biomaterials. Such biomaterials play an important role in the development of 3D platforms which along with being able to deliver the stem cells, they can also control stem cell behaviour by presenting appropriate biochemical cues. Biomaterial scaffolds can provide a 3D framework for the stem cells to propagate, differentiate and allows for remodelling of the surrounding

matrix upon action by the cells to form an implantable functional organoid. Numerous biomaterial scaffolds have been designed to drive the stem cells toward a particular lineage. For example, 3D systems were able to enhance the osteogenic (Sun et al. 2014), myogenic (Liu et al. 2012), neural (Cheng et al. 2013) and chondrogenic (Dvorakova et al. 2013) differentiation of various stem cells. In addition, these 3D platforms can also aid in forming stronger cell-cell and cell-matrix interactions. Further, the interactions of biomaterials with biomolecules have also been exploited to encapsulate various growth factors for presenting appropriate biomolecular cues to control stem cells (Fan et al. 2008). Because of all these advantages, 3D biomaterial scaffolds have become the basis for most of the stem cell therapy strategies of late. Often, such scaffolds are designed to allow for stem cell delivery through less invasive methods such as by injectable means.

Biomaterials used to fabricate the 3D culture platforms can be broadly classified into two types – natural and synthetic. Materials will have to be chosen according to the existing requirements of a specific application at hand. Properties of the biomaterials such as fluid transport, material degradation, surface chemistries for cell adhesion, mechanical strength, interaction ability with cells to induce signals, interactions with biomolecules for their controlled delivery need to be taken into consideration while choosing a biomaterial for any application (Dawson et al. 2008). Overall success of a stem cell therapy associated with biomaterial scaffolds would hinge upon the above mentioned material properties as they can determine the fate of many key processes such as nutrient diffusion, matrix remodelling, cell adhesion and differentiation. A large variety of biomaterials both natural and synthetic have been employed for stem cell cultures which are discussed in the following sections.

2.3.1. Synthetic biomaterials

Polymers and ceramics are two of the widely used synthetic matrices for stem cell therapies. While various types of polymers have been employed for many different types of tissue regenerative applications, ceramics such as calcium

phosphates are mainly employed for bone regeneration. Ceramics can provide higher mechanical strength and also have shown to enhance mineralization and matrix formation along with integrating well with the bone (Yuan et al. 2001, Arinzeh et al. 2005). On the other hand polymeric materials have been employed both for soft and hard tissue regeneration using stem cells. Polymers such as polyethylene glycol (PEG), polyglycolic acid (PGA), polylactic acid (PLA), polylactide-co-glycolide (PLGA), polycaprolactone (PCL) are some of the widely used ones for stem cell therapies. All these polymers are usually degradable in nature by hydrolysis and their degradation products formed are able to be physiologically removed. In addition, the main advantage of using synthetic polymers is that their degradation rates can be precisely controlled by manipulating the polymer chemistry and molecular weights (Lyu et al. 2009). Further, properties of these polymeric 3D scaffolds such as porosity, mechanical properties etc. can also be effectively tuned to fulfil the requirements of a specific application (Saha et al. 2007). However, although culture matrices made of synthetic materials provide good control over their physico-chemical properties, they are not bioactive by nature. Most of these matrices need to undergo biological or chemical modification to support cell culture and elicit a favourable cellular response. On the contrary, natural materials extracted from ECM are supportive of cell culture and are biologically active without the need for any modifications.

2.3.2. Natural biomaterials

ECM present in human bodies mainly consists of components such as collagen, fibrinogen, hyaluronic acid, heparin sulphate, chondroitin sulphate, glycosaminoglycans etc. Hence, most of these materials extracted from the ECM of different animal sources have been tried as culture platforms for culturing stem cells under *in vitro* conditions in order to mimic and create an *in vivo* like environment. Some other materials such as cellulose, chitosan and silk fibroin which are extracted from plants, other animals or insects have also been employed to fabricate 3D scaffolds for stem cell culture and differentiation. Although natural materials are very good at providing an *in vivo* mimicking culture

platform, they are also associated with some disadvantages. As most of these materials are extracted from animal sources there is chance for transmission of pathogens from animals to humans. Other drawbacks of natural materials include the difficulty in their purification and lack of complete control over their physico-chemical properties. However, lately many of these concerns have been addressed because of their commercial availability by many chemical companies which have set up standard protocols for the production of these materials with reproducible properties and with no carryover of pathogens.

Most typical of the commercially available natural materials are collagen and its derivatives. Collagen is the most abundant protein in the ECM of our body and thus 3D scaffolds made of collagen or its derivatives were believed to closely simulate the body conditions. In total, twenty seven collagen types have been identified till now (Koide 2007) among which type I collagen is the most abundant and widely employed one for biomedical applications. It was also found that very few people possess humoral immunity against type I collagen and vulnerability of a patient to it can be easily found out by a serological test before implantation (Parenteau-Bareil et al. 2010). Along with mimicking the native ECM, the chemical nature of collagen has also made it suitable for crosslinking using various chemical agents (Drury et al. 2003). This property of collagen permits for tailoring the mechanical and degradation properties of collagen based scaffolds as per specific requirements, and thus has attracted much attention for various biomedical applications including stem cell therapies.

Collagen has been widely used to fabricate different types of scaffolds such as hydrogels (Egawa et al. 2011), microspheres (Hui et al. 2008), nanofibers (Shih et al. 2006) etc. which were used extensively for various stem cell associated applications. In addition, over last decade or so, many collagen based tissue engineering products have been developed and commercialized by various companies (Malafaya et al. 2007). For example, a product named Apligraf®, which is a bilayered collagen gel seeded with fibroblasts and keratinocytes has been commercialized by Organogenesis in USA as artificial skin and got its FDA approval in 1998. Similarly, inFUSE® Bone Graft has been commercialized by

Medtronic Sofamor Danek which is a collagen sponge that acts as carrier for BMP-2 for spinal fusion. Angiotech Pharmaceuticals, Inc. in Canada has commercialized a composite made of porous hydroxylapatite, tricalcium phosphate and type I collagen under the name of Collagraft®. Many other collagen based products have also been commercialized or are under development by various companies for a wide range of applications including cosmetic products, skin replacements, bone and periodontal tissue grafts (Malafaya et al. 2007). All these examples which have been approved for clinical usage highlights the immense potential of collagen based scaffolds, firstly as promising biomaterials for several tissue regenerative applications and secondly for its translational ability to clinical stage.

Another prime derivative of collagen that has been widely used in tissue engineering is gelatin. Gelatin is a natural polymer obtained from collagen upon acid or alkaline processing. Gelatin is biodegradable, biocompatible and has been in regular usage for pharmaceutical and medical applications since long time. Its biosafety has been well proved from its usage as a plasma expander, as a component in drug formulations and also as a sealant in vascular prosthesis (Young et al. 2005). Gelatin also carries the key cell adhesion moieties, present in collagen and thus supports cell attachment and proliferation. Depending on the fabrication method employed two types of gelatin can be obtained (Figure 2.2), both of which are commercially available. Alkaline processing of collagen would yield a negatively charged acidic gelatin and an acidic treatment of collagen would give positively charged basic gelatin. Both types of gelatin are suitable for cell culture and basically differ in the electrostatic and physical properties. Depending on the application the type of gelatin need to be chosen appropriately. The different varieties of gelatin scaffolds used for stem cell applications and the parameters that determine the choice of acidic or basic gelatin is elaborated below.

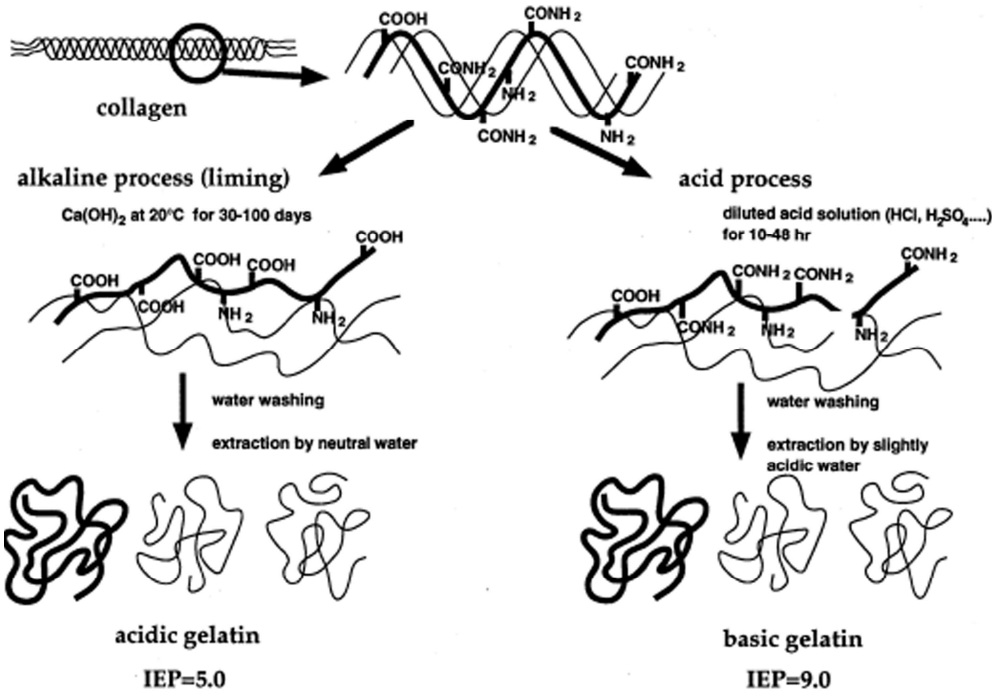

Figure 2.2 Collagen processing for acidic and basic gelatin preparation. Alkaline processing of collagen would yield a negatively charged acidic gelatin and an acidic treatment of collagen would give positively charged basic gelatin. Depending on the requirements of a specific application either type of gelatin can be chosen. For example, negatively charged acidic gelatin can be used to encapsulate positively charged basic biomolecules and vice-versa. Reproduced from (Ikada et al. 1998) by permission of Elsevier. Copyright © 1998, Elsevier.

Different types of scaffolds such as hydrogels, nanofibres and microspheres have been fabricated using gelatin alone or in combination with other biopolymers for tissue engineering applications (Kimura et al. 2003, Li et al. 2006, Liu et al. 2009, Hirai et al. 2013). Gelatin has a sol-gel transition of around 20 ^{o}C, and hence for gelatin scaffolds to keep their structure intact at body temperatures they need to be crosslinked. A variety of crosslinking agents have been tried such as glutaraldehyde, diisocyanates, carbodiimides, genipin etc. Alternatively, gelatin can be modified with methacrylate and can be crosslinked using UV in the presence of a photoinitiater (Lin et al. 2013). Many gelatin scaffolds have been previously employed for stem cell culture, differentiation and delivery. For example, gelatin/poly(ethylene glycol) biomatrices have been developed to deliver MSCs for wound healing (Xu et al. 2013). Scaffolds formed with gelatin and β-tricalcium phosphate or hydroxyapatite have been used for bone

regeneration using stem cells (Takahashi et al. 2005, Eslaminejad et al. 2007, Bernhardt et al. 2009). Electrostatic interactions of gelatin with the biomolecules can be used for encapsulation of various growth factors in gelatin scaffolds. Due to such electrostatic interactions gelatin can form strong poly-ion complexes with the oppositely charged biomolecules (Young et al. 2005). Negatively charged acidic gelatin can be used to encapsulate positively charged basic biomolecules and vice-versa. Thus depending on the kind of biomolecular cues that need to be presented for regulating stem cell behaviour, appropriate type of gelatin can be chosen. Gelatin microspheres have been well known as the most suitable of gelatin based scaffolds for control release of biomolecules.

2.3.3. Gelatin Microspheres

Microsphere scaffolds in general have certain unique features and advantages compared to other scaffold types. They tend to get their 3D nature through the curvature of the spheres which seem to have an enhanced effect on various cellular processes such as proliferation and differentiation (Schmidt et al. 2011). They can also be used as effective drug delivery vehicles and can control release various biomolecules. Thus microsphere scaffolds can usually be employed as delivery vehicles for both cells and growth factors simultaneously (Chen et al. 2010). This kind of an approach can be very beneficial for tissue regeneration as it can aid in three different ways. The microspheres can be packed together to form organoid constructs of required shape and size and depending on the material used for microsphere fabrication, they can aid as temporary replacements for the lost ECM of the damaged tissue. The cells and growth factors that are delivered using microsphere scaffolds can aid in regeneration by replenishing the lost functional cells and biomolecular signals within the tissue. Certain growth factors such as hepatocyte growth factor can even aid in recruiting the endogenous stem cells from the adjoining tissues (van de Kamp et al. 2013). Control release of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) can take part in the vascularization of the tissue which is crucial for proper supply of nutrients all over the tissue (Lovett et al. 2009).

Microsphere scaffolds made of gelatin termed as gelatin microspheres (GMs) are well known for their drug delivery abilities for various disease conditions (Jian Wang 2000, Vandelli et al. 2001, Nakase et al. 2002). GMs have also been successfully employed previously in various clinical trials (Nitta et al. 2009, Toyama et al. 2012) and thus regenerative systems made from them can have a better chance to succeed at clinical level compared to many other commercially available microcarriers. Recently they have also been employed for various tissue engineering applications and proven successful for delivering growth factors to enhance chondrogenic differentiation of MSCs (Fan et al. 2008). In this thesis, we employed GMs for enhancing the regenerative properties of ADSCs by forming cell-microsphere constructs. Microsphere scaffolds are also known to form strong cell-microsphere constructs which enhances the cell-cell and cell-matrix interactions (Zhu et al. 2007a, Zhu et al. 2007b, Zhu et al. 2008). Such interactions play a key role in tissue regeneration (Chen et al. 2012) and further shown to have improved the biological functions of stem cells (Hayashi et al. 2011). In a similar effort, in this thesis we studied if such strong cell-cell and cell-matrix interactions in the cell-microsphere constructs formed by ADSCs and GMs will enhance the biological functions of ADSCs which can further lead to accelerate the tissue regeneration process.

Figure 2.3. A schematic representation of gelatin microsphere fabrication and cell seeding.

2.4. Injectable delivery systems for stem cell therapy

Two kinds of approaches are primarily under consideration for stem cell therapies – (i) injection of stem cells with aqueous media to the wound site and (ii) injection of stem cells using 3D delivery systems. Direct injection of stem cells alone has previously caused cell death in certain instances (Guerette et al. 1997, Emgard et al. 2003) and proven ineffective for tissue regeneration (Lee et al. 2008). Injection of stem cells using 3D delivery vehicles which can provide cell adhesive sites were able to avoid cell death and also able to localise high densities of stem cells at the site of injury (Zakharova et al. 2010, Parisi-Amon et al. 2013). Such delivery vehicles can also provide a unique stem cell niche which can modulate stem cell response. Along with inducing stem cells to differentiate into a certain lineage of the damaged tissue, the micro-architecture of 3D systems can also regulate the growth factor secretion profiles of the stem cells (Guilak et al. 2009) which can aid in the tissue regeneration through paracrine signalling (Ratajczak et al. 2012).

Other advantages of such injectable delivery systems from a clinical perspective include ease of administration with significant reduction in treatment time, cost and patient morbidity along with smaller scar sizes and faster recovery (Fuchs 2002). Other than cell delivery, such injectable scaffolds can also be utilized as fillers for scar corrections, embolization agents, and also as in house biosensors (Munarin et al. 2012). Due to all these reasons, injectable delivery systems which can be employed with minimally invasive surgeries are becoming more attractive in the medical field for cell and drug delivery applications.

The effect of micro-architectural niche which include mechanical and biomolecular cues provided by these injectable delivery systems on the stem cell behaviour will be discussed in the following sections.

2.4.1. Effect of mechanical cues on stem cells

Stem cells can sense and respond to the physical micro-environment surrounding them. MSCs were found to be the most receptive stem cells which regularly encounter various kinds of physical forces inside the body, such as hydrostatic pressure, diffusive mass transport, shear stress etc. which drive them towards proliferation or differentiation and play a crucial role in the development of various tissues (Higuera et al. 2012). MSCs have the ability to differentiate into a wide variety of cell types ranging from neurons to osteocytes depending on the matrix stiffness (Engler et al. 2006). Matrices which are soft tends to induce neural lineage while stiffer and rigid matrices induces myogenic and osteogenic lineages respectively (Engler et al. 2006). This kind of a signalling from the matrix to the cells happens through large macromolecular assemblies of integrins called focal adhesions. Focal adhesions act as mechanical links between the surrounding matrix and the cytoskeleton of the cells and aid in transforming mechanical signals into biochemical signals by triggering various mechano-transduction pathways (Sun et al. 2012). Such biochemical signals then gets propagated to the cell nucleus which further regulates the stem cell behaviour by effecting the gene expression (Wang et al. 2009b).

The role of mechanical cues in determining stem cell fate has been exploited in various stem cell based tissue regenerative strategies. For example, mechanical properties of collagen-hyaluronic acid composite hydrogel scaffolds were tailored to direct the differentiation towards neuronal cells or glial cells for neural tissue regeneration (Her et al. 2013). Polyacrylamide gels conjugated with decellularized human lipoaspirates have been employed to stimulate adipogenesis of human ADSCs in the absence of any adipogenic growth factors by mimicking the native stiffness of adipose tissue (Young et al. 2013). Many other studies have also successfully demonstrated the induction of a specific cell type by providing with appropriate mechanical cues through injectable scaffolds (Ghosh et al. 2007).

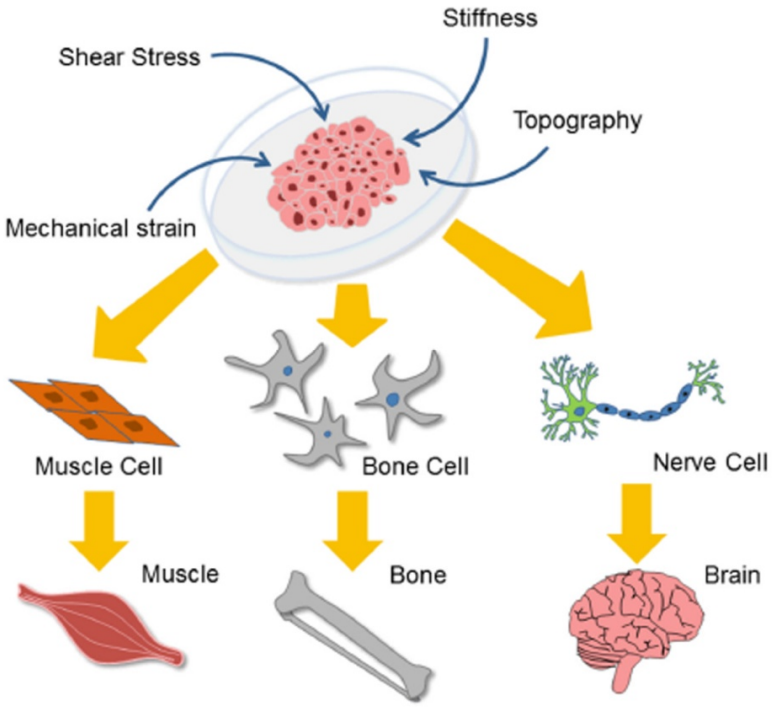

Figure 2.4 A schematic figure showing the effect of various biomechanical cues on stem cell behaviour. Various mechanical cues such as mechanical strain, shear stress, stiffness and topography seem to act in a synergistic fashion to regulate stem cell behaviour. Reproduced from (Kshitiz et al. 2012) by permission of The Royal Society of Chemistry. Copyright © 2012, The Royal Society of Chemistry.

2.4.2. Effect of biomolecular cues on stem cells

Along with mechanical cues, biomolecular cues also play an important role in determining the fate of the stem cells. During the initial weeks stem cells respond to the soluble induction factors and later on, the matrix elasticity drives the induction towards a certain lineage (Engler et al. 2006). Various types of biomolecules are being employed for different applications ranging from vascularisation to recruiting endogenous stem cells. VEGF is a well known angiogenic growth factor which can also induce endothelial differentiation in MSCs (Wang et al. 2013b). Chemokines such as stromal cell derived factor 1, can

recruit endogenous stem cells at the injury site and aid in the wound healing process (Imitola et al. 2004, Otsuru et al. 2008). At present, growth factors/cytokines are the most widely used biomolecular cues in tissue engineering strategies to induce different types of stem cells into all most all cell types of human body. In addition, cell adhesive motifs are also being employed by conjugating them with synthetic matrices to make them suitable for stem cell attachment and further to regulate the stem cell properties such as cell shape, migration and differentiation (Bacakova et al. 2004).

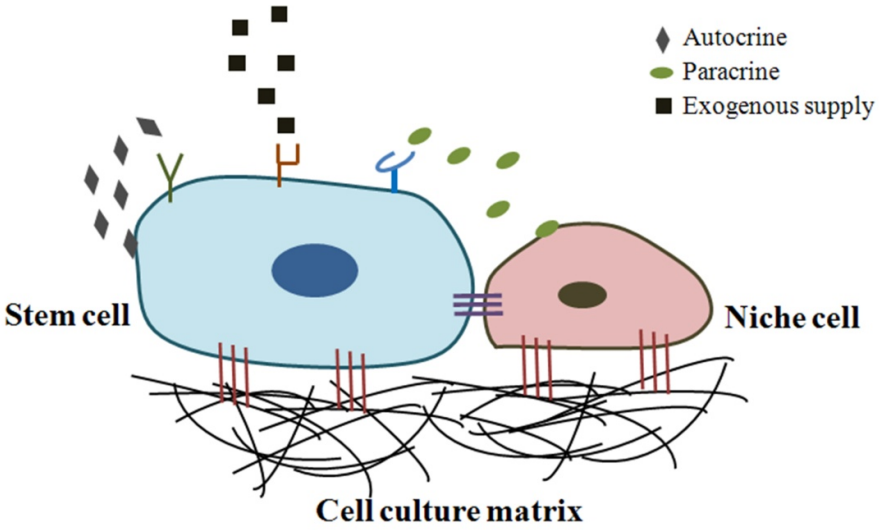

Figure 2.5 A schematic showing various biomolecular cues that are present in a stem cell niche that determines stem cell fate.

Hydrogels and microspheres are the two widely used 3D scaffolds which are well known for providing mechanical as well as biomolecular cues and can also be employed as injectable stem cell delivery systems are discussed in the following sections.

2.4.3. Hydrogels for stem cell therapy

Hydrogels are 3D scaffold materials which are made of hydrophilic polymers and are known to absorb high amounts of water. These matrices can closely resemble

physiological conditions with a proper selection of a biomimetic material which have tunable mechanical properties as well as high water content. They can be fabricated in different forms such as micro/nanoparticles or in the form of soft gels which can be injected in a liquid form and can gel *in situ* at the wound site (Hoare et al. 2008). This property of *in situ* gelling makes them an ideal choice to be used as fillers as they can take the appropriate shape of the defect site. They can also encapsulate stem cells and growth factors (Hwang et al. 2013a) and thus be effectively employed for stem cell therapies. A solution of gel precursors, stem cells and biomolecules can be injected into the body using a syringe and allowed to gel at the injury site. Once gelled, the 3D construct can provide appropriate chemical, mechanical, and biomolecular cues to enhance the regenerative potential of the encapsulated stem cells. The growth factors either encapsulated or secreted by the stem cells can also take part in the tissue regeneration either by recruiting endogenous stem cells or by improving vasculature through paracrine means.

Suitable materials need to be selected for fabrication of hydrogels for stem cell therapy based on the following criteria (Li et al. 2012). Firstly, the material should be biocompatible and biodegradable without release of any toxic by products upon degradation. Secondly, the viscosity of the materials should be reasonably low for them to be smoothly injectable and should be able to make a sol-gel transition under physiological conditions at appropriate gelation rates. Thirdly, the mechanical properties and degradation rates of the material should be tunable to fabricate them in compliance with the requirements of a specific application. Fourthly, it will also be advantageous if they can support controlled release of growth factors over desirable periods. Fifthly, the gel construct formed should have an appropriate porous structure for cell migration and also to support the exchange of nutrients as well as removal of waste.

Polymeric materials of both natural and synthetic origin have been employed previously for developing such injectable stem cell delivery systems. Natural polymers including chitosan, hyaluronic acid, alginate, collagen, gelatin, heparin and fibrin have been employed (Lee et al. 2001). Among the synthetic polymers,

peptides which can self assemble to form hydrogels have been employed but they are often expensive. Derivatives and co-polymers of poly(acrylic acid), poly(vinyl alcohol) (PVA), PEG and PCL have also been employed for making hydrogels for stem cell applications (Lee et al. 2001).

2.4.4. Microspheres for stem cell therapy

Microspheres have been used for the stem cell delivery in two ways – microencapsulation and microcarriers. In microencapsulation, cells are encapsulated inside the microspheres and in microcarrier cultures, cells are cultured on the surface of the microspheres. Both the approaches are discussed below.

Microencapsulation is a strategy which is usually employed when it is necessary to strictly separate the cells from the outside environment. For example, it can be used for cell delivery across immunological barriers (Jeon et al. 2006). Microencapsulation aids in avoiding the cells to come into contact with the host's immune system and thus can subside immunological issues. This approach also avoids the use of immunosuppressive drugs and also allows choosing the cells from a large variety of sources including allogenic and xenogenic sources (Zhang et al. 2008). However, escape of allogenic or xenogenic cells from the microcapsules due to excessive cell proliferation or degradation of the capsule might expose them to the host's immune system which might then attack those cells. Microencapsulation has also been employed for stem cell delivery using microcapsules termed as "artificial cells" which are capsules encapsulated with stem cells surrounded by strong and thin multilayer membrane components for nutrient transport (Paul et al. 2009). Materials employed for fabricating microspheres for this approach are usually hydrophilic polymers that form hydrogels. This permits the cells to be encapsulated in a hydrated environment which supports cell proliferation and migration. However, the main challenge in this strategy is the seeding of the cells into microspheres. If the cells are added to the precursor solution before fabricating microspheres, the cells have to bear the

harsh processing steps which might severely reduce the cell viability. Another approach is to seed the cells on the surface and allow the cells to migrate into the microspheres through the pores, which limits the uniform distribution of cells throughout the scaffold and will not permit optimal cell seeding densities.

In addition to microencapsulation, microsphere scaffolds can also be used as microcarriers for cell delivery by culturing the cells on the surface of microspheres. Such an approach will also allow to simultaneously encapsulate growth factors in the microspheres and controlled release them to present appropriate biomolecular cues to the cells cultured on the microsphere surface. For example, heparin crosslinked chitosan microspheres have been employed to deliver neural stem cells along with bFGF for central nervous system repair (Skop et al. 2013). This approach also forms cell-microsphere aggregates with strong cell-cell and cell-matrix interactions which proves to be crucial for tissue regeneration (Bratt-Leal et al. 2011). However, disadvantage of this kind of an approach is as the cells are exposed directly to outside environment, the shear forces during injection might effect the cells and might also detach the cells from microspheres which limits their usage as injectable delivery systems. In addition, maintaining the cell-microsphere constructs at the site of injury has also been found to be challenging (Pannek et al. 2001, Lemperle et al. 2004).

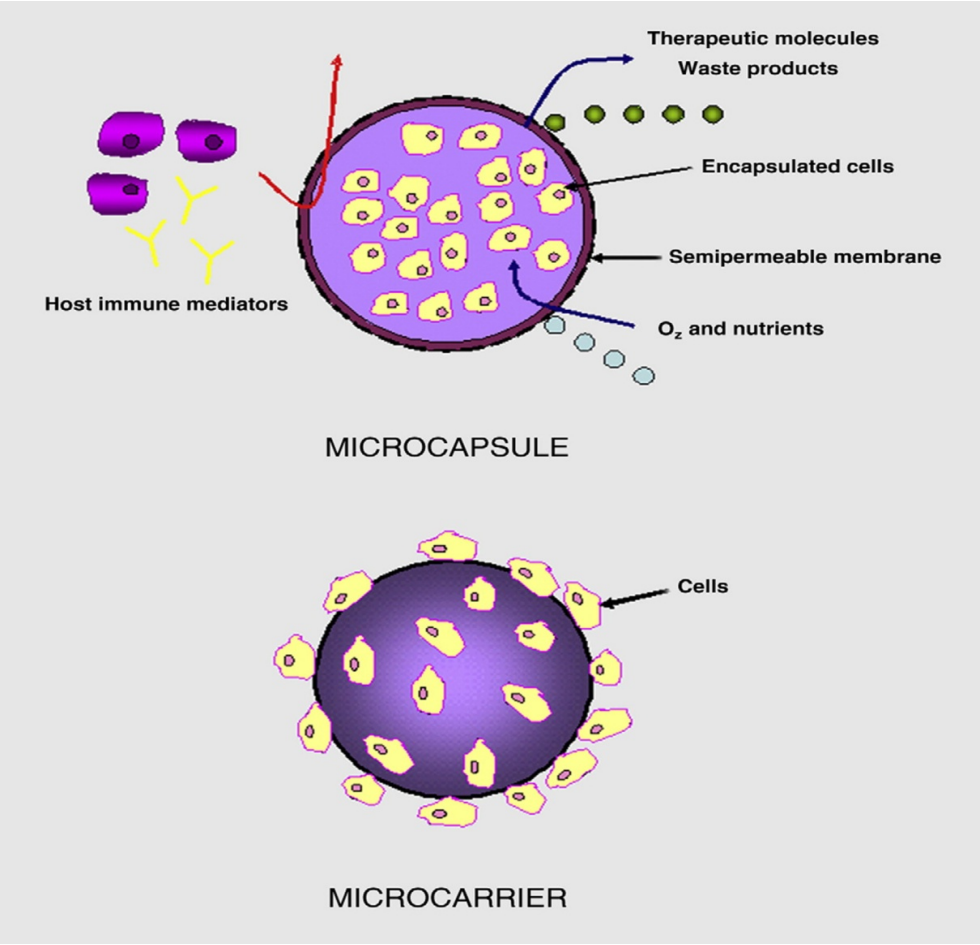

Figure 2.6 A schematic showing microcapsule and microcarrier technologies using microspheres. Microencapsulation is employed when it is necessary to separate cells from outside environment. For example, it is used to prevent the cells from getting exposed to immune system of the recipient. Microcarriers, on the other hand, allow cell culture on their surfaces and forms cell-microsphere constructs with strong cell-cell and cell-material interactions which are crucial for tissue regeneration. Reproduced from (Hernandez et al. 2010) by permission of Elsevier. Copyright © 2010, Elsevier.

2.4.5. Hydrogel-Microsphere composite scaffolds

Although both hydrogels and microspheres have been independently employed for stem cell delivery applications on many occasions as indicated in the above sections, they are also associated with some drawbacks which need to be overcome. For example, the mechanical strength of the traditional hydrogels is poor. Traditional approaches of crosslinking have improved their mechanical

strength but the crosslinking methods employed are often toxic to cells and thus effects the cell viability for *in situ* cell encapsulation. On the other hand, although microspheres have been proved effective as drug and cell delivery vehicles, maintaining them at the injury site seems challenging. In some instances, they were found in distant organs from where they were injected (Pannek et al. 2001). This kind of migration of cell-microspheres might lead to deleterious effects in other tissues.

To overcome the issues associated with both these systems, integrated delivery systems can be developed by a combination of these two techniques. Cells can be cultured on the surface of the microspheres and can be encapsulated in a hydrogel environment. The hydrogel network provides a 3D microenvironment for cells to proliferate and migrate and microspheres can be used as depots for growth factor delivery. The soft hydrogel surrounding the cell-microsphere constructs will also facilitates a minimally invasive means of delivery along with permitting for *in situ* gelation and cell encapsulation. Once gelled, the hydrogel can aid in maintaining the cell-microsphere constructs at the wound site and can also regulate the stem cell behaviour by providing with suitable mechanical and biomolecular cues. For example, PLGA microparticles encapsulated in collagen hydrogels (DeVolder et al. 2012) and TGF-β3 loaded alginate microspheres encapsulated in hyaluronic acid hydrogels (Bian et al. 2011) have been previously employed for bone and cartilage regeneration respectively using MSCs. Sequential delivery of growth factors can also be attained by loading different growth factors in hydrogel matrix and in microspheres (Kim et al. 2012). However, such an approach of hydrogel-microsphere composite systems for stem cell delivery has been relatively less studied. As discussed in the above sections 2.3.2 and 2.3.3, collagen hydrogels and GMs have been widely used for stem cell based tissue engineering applications and have high degree of potential to get translated to clinical level. Hence, in this thesis we examined the effect of such a composite scaffold fabricated by incorporating GMs in collagen hydrogels on the behaviour of ADSCs.

CHAPTER 3

MATERIALS AND METHODS

A detailed description of all the materials and methods employed for conducting the work presented in this thesis will be provided in this chapter

3.1. Materials

Gelatin, acetone, glycine, hoechst 33258, ethylene di-amine tetra-acetic acid (EDTA), sodium chloride (NaCl), phosphate buffer saline (PBS), sodium hydrogen carbonate ($NaHCO_3$), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium hydroxide (NaOH), dexamethasone, indomethacin, isobutyl methyl xanthine (IBMX), Oil Red O, isopropyl alcohol, paraformaldehyde, ascorbic acid, β-glycerol phosphate, Alizarin red S, ammonium hydroxide (NH_4OH), nicotinamide, hoechst, phalloidin-tetramethylrhodamine B isothiocyanate (TRITC), ammonium chloride (NH_4Cl), bovine serum albumin (BSA), saponin, all qPCR primers, growth factor reduced matrigel, 24 well transwell culture plates, heparin and endothelial cell growth supplement were obtained from Sigma (USA). Olive oil was purchased from Wako chemicals (Japan). Glutaraldehyde was purchased from Merck (USA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin and antibiotics for cell culture were obtained from Hyclone (USA). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and bFGF ELISA kit were purchased from Peprotech (USA). RNeasy mini kit for RNA extraction bought from Qiagen. Maxima first strand cDNA synthesis kit obtained from Fermentas, Thermo fisher (USA). SYBR FAST Biorad qPCR master mix was purchased from Kapa Biosystems (USA). Human umbilical vein endothelial cells (HUVEC) and F-12k medium were obtained from the American type culture collection (ATCC) (USA). Collagen type I obtained from Advanced biomatrix (USA). Collagenase type I and insulin were purchased from Gibco, Life technologies (USA). Antibodies for albumin, alpha-fetoprotein and cytokeratin 18 along with alkaline phosphatase assay kit were obtained from Abcam (USA). Pierce BCA protein assay kit for protein estimation was purchased from Thermo scientific, (USA).

3.2. Fabrication and Characterization of cell-microsphere (ADSC-GM) constructs

3.2.1. Gelatin microsphere fabrication and characterization

Gelatin microspheres (GMs) were fabricated using a water-in-oil emulsion method (Zhu et al. 2008). Briefly, 4 g of gelatin was dissolved in 20 mL of water and heated up to 60 ^{o}C. 200 mL of olive oil was heated up to 40 ^{o}C. Gelatin was then added drop-wise into the olive oil, while stirring at 420 rpm with a mechanical stirrer (RW20; Ika Labortechnik, Staufen, Germany). The water-in-oil (w/o) emulsion was stirred for 10 min before being immersed into an ice bath to maintain the temperature at 10 ^{o}C and stirred for a further 30 min. 60 mL of chilled acetone was then added and the mixture was stirred for another 1 h. The GMs were extracted from the olive oil by a series of centrifuging and washing with chilled acetone. Crosslinking was carried out by immersing the microspheres in 150 mL of 10 mM glutaraldehyde solution and stirred at 420 rpm for 12 h at 4 ^{o}C. Crosslinked microspheres were washed with deionized (DI) water and then suspended in 50 mM glycine solution to block the unreacted aldehyde groups for 2 h at room temperature. The microspheres were then washed with acetone and air dried. Thus obtained GMs were stored at -20 ^{o}C for future use.

The GMs were characterized for their sizes using an optical microscope and their surface morphology using a scanning electron microscope (SEM) (JSM-5600VL; JEOL, Tokyo, Japan). As GMs tend to swell by absorbing water, the sizes of microspheres were measured both in dry and in wet state, after saturation with sterile DI water for 3 h. Images were taken using a digital camera attached to the optical microscope and the sizes were analyzed by measuring the diameters of the microspheres using image pro software. For imaging under SEM, dry microspheres were mounted onto brass stubs using a two-sided adhesive tape and platinum coated for 40 s using Auto Fine Coater (JFC-1300; JEOL).

3.2.2. ADSCs isolation and culture

ADSCs were isolated from adipose tissues obtained from patients undergoing liposuction with informed consent and approval from the Institutional Review Board, National University Hospital, Singapore. The obtained adipose tissues were processed for ADSCs isolation using an established protocol (Leong et al. 2005). Briefly, the tissues were washed with phosphate buffer saline (PBS) on a separating sieve and treated with 0.075% collagenase type I for 1 h at 37 ^{o}C under shaking. Cells were pelleted out by centrifugation at 1200 x g, 4 ^{o}C for 10 min and plated in tissue culture flasks along with growth medium (DMEM containing 10% fetal bovine serum and 1% antibiotics). After the cells were attached, the medium was removed, washed with PBS and replaced with fresh medium. The cells were cultured to 80% confluency and then passaged for cell expansion. ADSCs of passages 2 to 4 have been used for the experiments in this study.

3.2.3. Cell seeding on gelatin microspheres

GMs crosslinked with 10 mM glutaraldehyde were sterilized with 70% ethanol followed by complete washing with sterilized PBS. For differentiation experiments, the microspheres were then transferred to 12 well plates at 10 mg per well and $5x10^4$ cells were then seeded onto the microspheres per well (i.e. $5x10^3$ cells per mg of microspheres). For cell proliferation experiments, the microspheres were transferred to 24 well plates at 2 mg per well and $1x10^4$ cells per well were subsequently seeded onto the microspheres.

3.2.4. Total DNA quantification assay

Total DNA quantification method was used to study the proliferation of ADSCs on GMs and on traditional 2D tissue culture dishes. ADSCs were cultured on the microspheres and on 2D over a period of 10 d and samples were collected on different time points for performing the assay. The culture samples were washed twice with PBS and the cells were lysed by incubating with ultrapure water followed by three freeze-thaw cycles. A solution of 1 μg/mL Hoechst 33258 in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.2 M NaCl was prepared and mixed

with the cell lysates to incubate in dark for 30 min. Subsequently fluorescent readings were measured using a microplate reader (Infinite M200, Tecan) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

3.2.5. Differentiation of ADSCs and characterization

Following differentiation media were used to differentiate ADSCs both in monolayer and on GMs into adipogenic, osteogenic and hepatic lineages.

For adipogenic differentiation, ADSCs were maintained in the adipogenic induction medium (Wall et al. 2007) comprising of growth medium supplemented with 1 µM dexamethasone, 5 µg/mL insulin, 100 µM indomethacin and 500 µM isobutyl methyl xanthine. Adipogenic differentiation was characterized by Oil Red O staining after three weeks of induction. To further confirm the differentiation, expression of adipogenic marker gene PPAR-γ was also analyzed using real time PCR.

For osteogenic differentiation, ADSCs were maintained in the osteogenic induction medium (Wall et al. 2007) comprising of growth medium supplemented with 50 µM ascorbic acid, 0.1 µM dexamethasone and 10 mM β-glycerol phosphate. After three weeks of induction the cells were fixed with 4% paraformaldehyde and stained with Alizarin red S to observe the presence of mineralized matrix deposition. Real time PCR was also performed to analyze the gene expression of a key osteogenic transcription factor gene, RunX2.

Hepatic differentiation was performed by culturing the ADSCs in serum free medium containing DMEM supplemented with 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF) for 48 h. For the next two weeks, ADSCs were cultured in DMEM supplemented with 20 ng/mL hepatocyte growth factor (HGF), 10 ng/mL bFGF and 4.9 mM nicotinamide (Talens-Visconti et al. 2007). After two weeks, the hepatic differentiation was characterized by studying the gene expression of albumin.

Presence of hepatic markers albumin, alpha-fetoprotein and cytokeratin18 was confirmed by performing immunofluorescence staining.

3.2.6. Oil Red O Staining

ADSCs differentiated towards adipogenic lineage both on 2D and on GMs were stained with Oil Red O for characterizing the differentiation. Prior to performing the staining, appropriate amount of Oil Red O solution was prepared. Firstly, a stock solution of 0.5% Oil Red O solution in isopropyl alcohol is prepared, which can be stored up to 3 months at room temperature upon protection from light. Working solution of Oil Red O was then freshly prepared by mixing 3 parts of stock solution to 2 parts of PBS. The working solution was then thoroughly mixed and left for 10 min. This solution was finally filter sterilized and used within 2 hours of preparation.

Once the Oil Red O working solution was prepared, the differentiation media was removed from all the wells and the samples were washed twice with PBS. ADSCs were then fixed using 4% paraformaldehyde for 1 h at room temperature and the samples were rinsed with PBS twice. 2 mL of Oil Red O working solution was then added to each well and incubated for 20 min at room temperature. The stain solution was removed and the samples were washed again with PBS twice. All the samples were finally examined under an optical microscope for visualization of lipid droplets inside differentiated ADSCs and their images were taken using a digital camera attached to the microscope.

3.2.7. Alizarin red staining

ADSCs differentiated towards osteogenic lineage both on 2D and on GMs were stained with Alizarin red S for characterizing the differentiation. Prior to performing the staining, appropriate amount of stain solution was prepared. 1 g of Alizarin red S was dissolved in 100 mL of DI water and pH of the solution was adjusted to be between 4.1 and 4.3 using 0.1% ammonium hydroxide. This

solution was then filter sterilized and used for staining, which can be stored up to 3 months at room temperature upon protection from light.

Once the Alizarin red stain solution was prepared, the differentiation media was removed from all the wells and the samples were washed twice with PBS. ADSCs were then fixed using 4% paraformaldehyde for 1 h at room temperature and the samples were rinsed with DI water twice. 2 mL of Alizarin red stain solution was then added to each well and incubated for 20 min at room temperature. The stain solution was removed and the samples were washed again with DI water twice. All the samples were finally examined under an optical microscope for visualization of mineralized matrix depositions by the differentiated ADSCs and their images were taken using a digital camera attached to the microscope.

3.2.8. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from ADSCs cultured both on tissue culture plates and on GMs using Qiagen RNeasy mini kit. Concentrations of the extracted total RNA was determined using Nanodrop 2000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized from the extracted RNA using Maxima first strand cDNA synthesis kit (Thermo Scientific). The obtained cDNA was used for qPCR which was performed in triplicates by using SYBR FAST Biorad qPCR master mix (Kapa Biosystems). The primers used in the qPCR are given in Table 3.1. Relative gene expression was found out using double delta Ct method (Livak et al. 2001) using β-actin as housekeeping gene.

Table 3.1 Primer sequences used for qPCR experiments

Gene name		Primer sequence
Oct4	Forward	5'-GCAGCGACTATGCACAACGA-3'
	Reverse	5'-CCAGAGTGGTGACGGAGACA-3'
Sox2	Forward	5'-CATCACCCACAGCAAATGACA-3'
	Reverse	5'-GCTCCTACCGTACCACTAGAACTT-3'
Nanog	Forward	5'-CCTGTGATTTGTGGGCCTG-3'
	Reverse	5'-GACAGTCTCCGTGTGAGGCAT-3'
Rex1	Forward	5'-TGAAAGCCCACATCCTAACG-3'
	Reverse	5'-TATAACCGCTTTTGGGGTTG-3'
PPAR-γ	Forward	5'-TCAGGTTTGGGCGGATGC-3'
	Reverse	5'-TCAGCGGGAAGGACTTTATGTATG-3'
Runx2	Forward	5'-TTCATCCCTCACTGAGAG-3'
	Reverse	5'-TCAGCGTCAACACCATCA-3'
Albumin	Forward	5'-TGTTGCATGAGAAAACGCCA-3'
	Reverse	5'-GTCGCCTGTTCACCAAGGA-3'
β-actin	Forward	5'-CATGTACGTTGCTATCCAGGC-3'
	Reverse	5'-CTCCTTAATGTCACGCACGAT-3'

3.2.9. Immunofluorescence staining

The ADSCs differentiated both in monolayer and on GMs were fixed with 4% paraformaldehyde for 20 min. The samples were then washed with PBS, 100 mM NH_4Cl and again with PBS. Following this, cells were permeabilized in 0.1% W/V saponin for 15 min. Primary antibodies were diluted in blocking buffer consisting of 5% fetal bovine serum and 2% bovine serum albumin in PBS and were added to the culture samples for 1 h. The samples were subsequently washed thrice with PBS and incubated for 1 h with a solution containing secondary antibody, Hoechst and phalloidin diluted in blocking buffer. Finally, the samples were washed for six times with PBS and visualized under a confocal laser scanning microscope (CLSM) (C1 system, Nikon, Singapore).

3.2.10. *In vitro* HUVEC-matrigel assay

To test the *in vitro* pro-angiogenic activity of the ADSCs cultured on 2D and on GMs, HUVEC-matrigel assay was performed. HUVECs were co-cultured with ADSCs either cultured on 2D or on GMs and tube formation ability of HUVECs on matrigel was quantified. HUVECs alone without any co-culture with ADSCs were used as control. 24-well transwell culture plates were used for the co-culture experiments. HUVECs purchased from ATCC were maintained in growth medium consisting of F-12K medium supplemented with 0.1 mg/mL heparin, 0.05 mg/mL endothelial cell growth supplement and 10% fetal bovine serum.

For matrigel assay, ADSCs were initially cultured in the culture inserts either on 2D or on GMs for two days and conditioned media were collected. On day 3 the bottom wells of the transwell plates were coated with growth factor reduced matrigel and incubated for 30 min at 37 ^{o}C for gelation. HUVECs were trypsinized and seeded onto matrigel coated wells at a density of $5x10^4$ cells per well. The co-culture was performed for 24 h in medium containing both ADSC conditioned medium and HUVEC growth medium in 1:1 ratio. After 24 h, optical images were taken and quantitative measurements were made for tube lengths and number of branch points of the HUVEC tubules formed on matrigel using ImageJ software.

3.3. Osteogenic induction of ADSCs in Col-GM composite scaffolds

3.3.1. Fabrication of Col-GM scaffolds

GMs were fabricated using a water-in-oil emulsion method and crosslinked with 10 mM glutaraldehyde as described the section 3.2.1. Pure collagen gels were also synthesized using a previously described method (Liang et al. 2011). Briefly, pre-chilled bovine collagen type I solution (3 mg/mL, PureCol, Advanced Biomatrix), growth medium containing ADSCs (50,000 cells per 1 mL volume of gel) and reconstitution solution were mixed in a ratio of 8:4:1. The reconstitution solution

was a mixture of 0.26 M of sodium hydrogen carbonate, 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.04 N of sodium hydroxide. The mixture was subsequently incubated at 37 ^{o}C for 4 h to form 3D collagen hydrogels (Col). To form Col-GM scaffolds, ADSCs were first seeded onto GMs as described in section 3.2.3 at a concentration of 50,000 cells per 10 mg of GMs. This cell-microsphere mixture was first incubated in 200 µL of growth medium at 37 ^{o}C for 4 h for the cells to attach to the GMs. Then cell-microsphere constructs were mixed with the pre-chilled collagen type I solution and reconstitution solution as described above to form the Col-GM scaffolds. ADSCs seeded only on GMs were used as control groups in the experiments as required.

3.3.2. Rheological measurement of Col-GM scaffolds

Rheological properties of the scaffolds were measured using an AR-G2 rheometer (TA instruments, New castle, USA) using parallel plates of diameter 40 mm at 37 ^{o}C. Firstly, the scaffolds were formed by mixing respective pre-gel solutions and then added onto the rheometer. After a brief equilibration of the gels on the rheometer plates at 37 ^{o}C, rheological measurements were carried out. Dynamic strain sweeps were first carried out from 0.1% to 100% at an angular frequency of 1 rad/s to find out the linear visco-elastic region. Following that, the strain was fixed at 1% and frequency sweeps were performed over the frequency range of 0.1 – 10 rad/s.

3.3.3. Immunofluorescence staining

On various time points during a 10 d culture in growth medium, ADSCs cultured in Col-GM scaffolds were fixed with 4% paraformaldehyde for 20 min. The samples were then washed once with PBS, twice with 100 mM NH_4Cl and again with PBS. Following this, cells were permeabilized in 0.1% W/V saponin for 15 min. Hoechst and phalloidin-TRITC were diluted in blocking buffer consisting of 5% fetal bovine serum and 2% bovine serum albumin in PBS and were added to the culture samples for 1 h. Finally, the samples were washed for six times with

PBS and visualized under a confocal laser scanning microscope (CLSM) (C1 system, Nikon, Singapore).

3.3.4. Real-time quantitative polymerase chain reaction (qPCR)

Col-GM scaffolds on appropriate time points during the differentiation of ADSCs were first treated with collagenase type I solution (1,000 units/mL in PBS) at 37 ^{o}C for 1 h to break the collagen matrix. Total RNA was subsequently extracted from all the samples and qPCR was performed according to the protocol described in the section 3.2.8. The primer sequences used for the qPCR are given in Table 3.2.

Table 3.2 Primer sequences used for qPCR experiments

Gene name		Primer sequence
BMP2	Forward	5'-GGAATGACTGGATTGTGGCT-3'
	Reverse	5'-TGAGTTCTGTCGGGACACAG-3'
OCN	Forward	5'-CAAAGGTGCAGCCTTTGTGTC-3'
	Reverse	5'-TCACAGTCCGGATTGAGCTCA-3'
Runx2	Forward	5'-GTCTTACCCCTCCTACCTGA-3'
	Reverse	5'-TGCCTGGCTCTTCTTACTGA-3'
β-actin	Forward	5'-ATCATGTTTGAGACCTTCAA-3'
	Reverse	5'-CATCTCTTGCTCGAAGTCCA-3'

3.3.5. Encapsulation of bFGF in Col-GM scaffolds and *in vitro* release study

GMs were sterilized with 70% ethanol followed by complete washing with sterilized PBS and then were air dried for 5 h under sterile conditions. 100 µL of bFGF solution (1 ng/µL) was then added to 20 mg of such air dried GMs and were left for 5 h for the GMs to absorb the bFGF solution completely while swelling. These bFGF loaded GMs were then incorporated into collagen hydrogels fabricated as described above. For incorporating bFGF into collagen hydrogels, 100 ng of bFGF solution was prepared at appropriate concentration and mixed with collagen type I solution and reconstitution solution to form the hydrogel.

To study the release profile, bFGF loaded scaffolds were suspended in 2 mL of DMEM and incubated at 37 ^{o}C for 14 days. At set time points, the supernatant was collected and replaced with fresh DMEM. The concentrations of bFGF in the collected supernatants were analyzed using an ELISA kit (Peprotech) according to manufacturer's instructions.

3.3.6. Alkaline phosphatase (ALP) activity

ALP activity of all the Col-GM scaffolds was quantified on predetermined time points for assessing the osteogenic differentiation of ADSCs. A calorimetric assay kit (Abcam, Cambridge, UK) was used to evaluate the ALP activities. The kit uses p-nitrophenyl phosphate as substrate for ALP and its conversion to p-nitrophenol can be analyzed by measuring the absorbance at 405 nm. Osteogenic medium from all the samples was aspirated and washed with PBS. 300 µL of lysis buffer provided with the kit was then added directly to all the samples and were incubated for 15 min. After confirming the cell lysis by checking under microscope, the samples were centrifuged at 13,000 x g for 3 min at 4 ^{o}C to remove the cell and scaffold debris. The supernatants were subsequently collected and ALP activities were measured according to manufacturer's instructions. Total protein from all the samples was also quantified using Pierce BCA protein assay kit (Thermo scientific, Illinois, USA). ALP activities from different samples were further normalized with their respective total protein values.

3.4. Statistical analysis

All the data presented in this study represents mean ± standard deviation values of three experiments, unless otherwise stated. Statistical differences between groups were found using Student's t-test or One-way ANOVA. Differences with $P<0.05$ were considered as statistically significant and were represented with appropriate symbols.

CHAPTER 4

FABRICATION AND CHARACTERIZATION OF CELL-MICROSPHERE CONSTRUCTS FORMED WITH HUMAN ADIPOSE DERIVED STEM CELLS AND GELATIN MICROSPHERES

This chapter describes the strategy of cell-microsphere constructs (ADSC-GMs) as an in vitro 3D culture platform for human ADSCs and their properties such as proliferation, stemness, multi-lineage differentiation and pro-angiogenic potential. The work in this chapter addresses objective 1 described in chapter 1.This work has been published in Macromolecular Bioscience.

4.1. Introduction

Adipose derived stem cells (ADSCs) which have very similar characteristics to that of bone marrow mesenchymal stem cells (BMSCs) (Kern et al. 2006) are gaining increased interest because of their immense therapeutic potential evident from recent studies (Leong et al. 2006, Li et al. 2011, Lin et al. 2011, Tay et al. 2011, Choi et al. 2012, Hwang et al. 2013a). However, many of the studies involving ADSCs have been performed on two dimensional (2D) tissue culture dishes which do not resemble the *in vivo* microenvironment. In addition, direct injection of such cell suspensions cultured on 2D have proven to be inefficient for regenerating tissues due to poor engraftment and lack of control over cell distribution inside body (Lee et al. 2008, Mooney et al. 2008). In many instances, most of the transplanted cells die shortly after implantation (Guerette et al. 1997, Emgard et al. 2003). Dearth of cell attachment sites and existence of ischemic environment due to poor vascularization might be the reasons for such cell death. Microcarriers designed as injectable cell delivery vehicles which can provide cell attachment sites and also aid in vascularizing regenerative tissue can overcome this problem. Such microcarriers can be used to deliver both cells as well as biomolecules simultaneously (Chen et al. 2010). Zhu et al. have previously employed basic fibroblast growth factor loaded gelatin microspheres (GMs) for culturing human umbilical vein endothelial cells (HUVECs) in a similar effort to promote vascularization for tissue implants (Zhu et al. 2008). GMs are well established drug delivery vehicles (Young et al. 2005) which have also been studied for their suitability in various tissue engineering applications (Zhu et al. 2008, Baraniak et al. 2012, Leong et al. 2013). In this chapter, we report 3D GMs as viable platforms for tissue engineering with human ADSCs. To this end, we studied the role of GMs in controlling stemness, angiogenic and differentiation properties of ADSCs, all of which are important factors for tissue regeneration.

The regenerative capacity of ADSCs for clinical applications can be enhanced by promoting their stemness and angiogenic properties. Maintaining stemness improves the multi-lineage differentiation ability of ADSCs while pro-angiogenic

properties can be useful for vascularization of regenerating tissues. Recent studies have employed 3D spheroids for enhancing stemness, angiogenic and other functional properties of ADSCs as well as other cell types (Lin et al. 2008, Cheng et al. 2012, Laschke et al. 2013). Presence of stronger cell-cell interactions in spheroidal morphology was found to be the reason for such enhanced functional abilities. However, spheroids are associated with diffusional limitations and also lack cell-matrix interactions. Some other studies using microsphere scaffolds have shown that such strong cell-cell and cell-matrix interactions can also be generated by forming compact cell-microsphere constructs with superior control over cell behaviour (Khew et al. 2007, Zhu et al. 2007a, Zhu et al. 2007b, Zhu et al. 2008, Chen et al. 2012).

Thus the objective of this study is to investigate whether the ADSCs can similarly form such cell-microsphere constructs (ADSC-GMs) with enhanced tissue regenerative abilities suitable for direct clinical use. To achieve this objective, we conducted experiments to test the ability of ADSC-GM constructs for three different aspects of tissue regeneration. Firstly, maintenance of stemness properties in the ADSC-GM constructs was studied. Recent studies have shown that well known pluripotent marker genes such as Oct4, Sox2, Nanog and Rex1 play an important role in self-renewal and preserving differentiation abilities of ADSCs and BMSCs (Greco et al. 2007, Riekstina et al. 2009, Baer et al. 2010). Therefore, these four genes were selected to test the stemness properties and their gene expression was studied using quantitative polymerase chain reaction (qPCR) during the proliferation of ADSCs. Secondly, the multipotent differentiation abilities of ADSC-GMs were studied by inducing them towards adipogenic, osteogenic and hepatic lineages. Thirdly, pro-angiogenic ability of the constructs was also studied using an *in vitro* HUVEC-matrigel assay. HUVECs were co-cultured with either ADSC-GMs or ADSCs on 2D and the tube formation ability of HUVECs on the matrigel was quantified. This ADSC-GM construct strategy for stem cell culture and delivery can be a promising approach for the use of adult stem cells in regenerative medicine.

4.2. Results

4.2.1. Fabrication of Gelatin microspheres

All the GMs fabricated were found to be spherical with smooth surfaces as shown under SEM (Figure 4.1c). The optical microscope images of the GMs in dry (Figure 4.1a) and wet (Figure 4.1b) conditions show the swelling nature of these hydrogel microspheres. Sizes of the microspheres sampled from a single batch of microspheres were measured and found to be 103.8 ± 27.5 µm when dry and 145.7 ± 46.4 µm when swollen with a mean swelling ratio of 2.77.

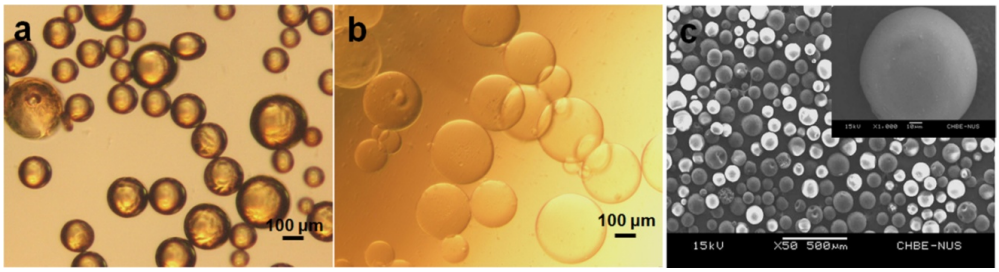

Figure 4.1 Optical microscope images of GMs in (a) dry and (b) wet condition. (c) SEM image of GMs showing the sphericity of the GMs and SEM image in the inset showing the smooth surface of the GMs.

4.2.2. ADSC culture and proliferation on gelatin microspheres

Figure 4.2a and b, show the optical microscope images of ADSC-GM constructs on day 3 and day 7 respectively. ADSCs seem to attach and spread well on the microspheres by day 3. Some of the elongated ADSCs tend to extend over individual microspheres bridging adjacent microspheres. By day 7, ADSCs became confluent over the microspheres, strengthening the bridges between microspheres to form large cell-microsphere clusters. To study the morphology of ADSC-GMs in detail, SEM (Figure 4.2c) and CLSM (Figure 4.2d) imaging of ADSC-GMs was performed after one week of culture. SEM images of ADSC-GMs show differences in surface morphology from the plain GMs shown in

Figure 4.1c which has a smooth surface. That could be due to the GMs being covered with cells and extracellular matrix secreted by the cells. The presence of cells and the extracellular matrix helps in holding the ADSC-GM construct as an integral organoid graft. Such a coordinated bridging at the cellular level will further lead to higher hierarchical integrity. From the CLSM images, actin filaments of ADSCs were seem to be stained with phalloidin-TRITC (red) and nucleus stained with Hoechst (blue). The image in Figure 2d clearly confirms the presence of ADSCs on microspheres and their spreading.

To further study the proliferation of ADSCs on GMs, total DNA quantification assay was performed for microsphere cultures (Zhu et al. 2007b). A calibration curve was used to obtain the cell numbers on different days of culture as presented in Figure 4.3a. The results show that cell numbers increase from day 1 to day 10 both on 2D and on GMs. ADSCs tend to proliferate faster on GMs as compared to 2D cultures as seen by their mean doubling times of around 2 days and 2.5 days respectively measured between day 3 and day 7 of the log phase. ADSCs can adhere better to GMs compared to the tissue culture plates because of the presence of cell adhesive moieties on gelatin and the GMs also provide a larger surface area compared to 2D cultures. These might be the reasons for the faster growth rate of ADSCs that is observed on GMs. The cell proliferation results obtained are in agreement with previous studies on microsphere scaffolds (Zhu et al. 2007a, Zhu et al. 2007b).

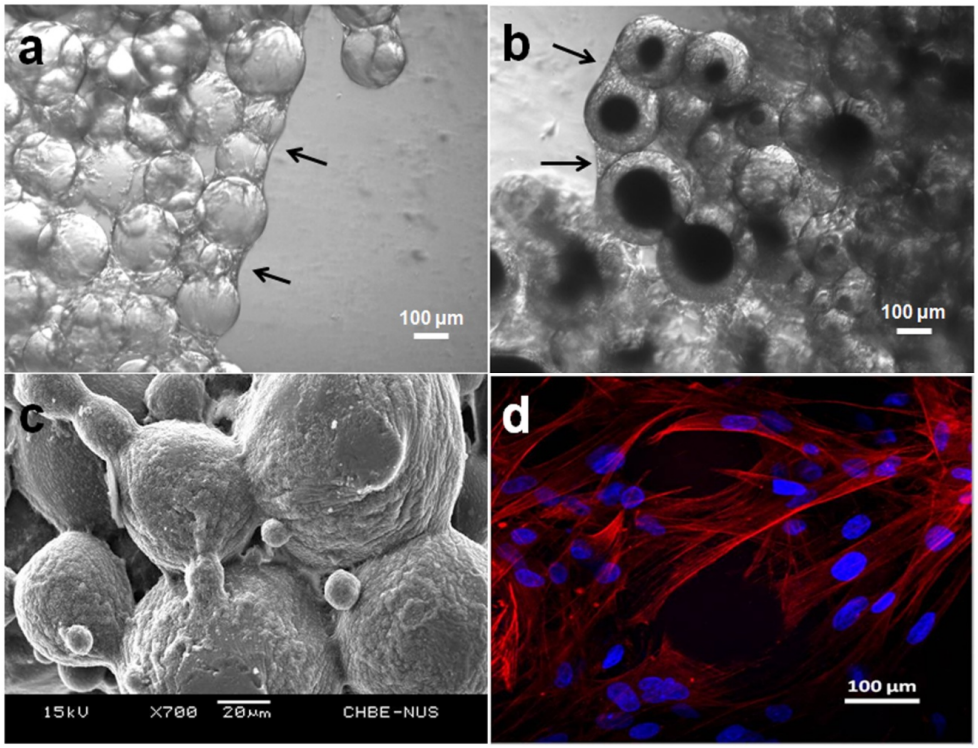

Figure 4.2 ADSCs cultured on GMs. Optical microscope images of ADSC-GMs on (a) day 3 and (b) day 7 of culture period. Black arrows showing the bridging of adjacent GMs by elongated ADSCs. (c) SEM and (d) CLSM images of ADSC-GMs on day 7. For CLSM image cell actin was stained with phalloidin-TRITC and nucleus with Hoechst.

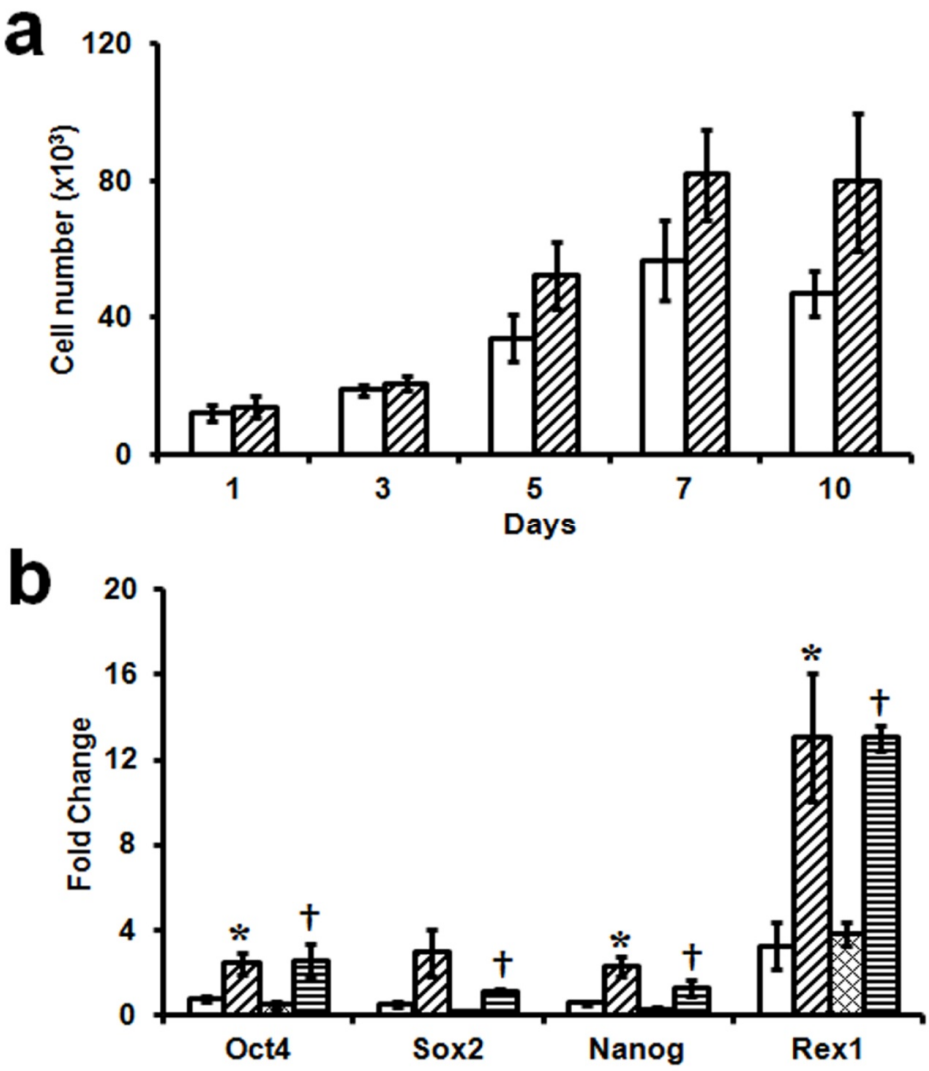

Figure 4.3 (a) Proliferation of ADSCs on 2D (▭) and on GMs (▨) studied using total DNA quantification assay. Differences in cell numbers on 2D and GMs were not found to be statistically significant. (b) qPCR fold change values measured relative to day 0 control for stemness marker genes Oct4, Sox2, Nanog and Rex1 of ADSCs cultured on 2D and GMs after day 3 and day 7. Error bars represent SD (n=3); *P<0.05 (student's t-test) compared to 2D group on day 3 and †P<0.05 (student's t-test) compared to 2D group on day 7.
(▭ 2D day 3; ▨ GMs day 3; ▩ 2D day 7; ▤ GMs day 7)

4.2.3. Expression of stemness marker genes on gelatin microspheres

We studied the expression of well-known stemness marker genes Oct4, Sox2, Nanog and Rex1 using qPCR for ADSCs cultured both in monolayer and on GMs. It was found that ADSCs cultured on GMs expressed significantly higher amounts of Oct4, Sox2, Nanog and Rex1 as compared to the monolayer cultures shown in Figure 4.3b. Gene expression of all four genes studied seems to be upregulated on day 3 compared to day 0 controls in ADSC-GMs with mean fold changes of 2.4, 2.9, 2.3 and 13.1 for Oct4, Sox2, Nanog and Rex1 respectively. In contrast on 2D culture plates, there was a down regulation of all genes except for Rex-1 which was upregulated by 3.2 fold. The expression levels of Oct4 and Rex1 on ADSC-GMs were maintained even after one week of culture with average fold changes of 2.6 and 13 compared to day 0 controls. However, there was a decline in the expression levels of Sox2 and Nanog on day 7 as compared to day 3, possibly due to culturing in serum containing medium. Optimizing the media conditions might further enhance the gene expression of these stemness genes (Baer et al. 2010). Our results demonstrate that ADSCs cultured on traditional 2D tissue culture dishes tend to lose their stemness marker gene expression which are in agreement with other studies (Park et al. 2010a). On the contrary, ADSC-GMs can overcome that problem by providing an artificial stem cell niche which preserves the expression of stemness genes.

4.2.4. Adipogenic and osteogenic differentiation of ADSCs

ADSCs cultured both in monolayer and on GMs were able to differentiate into adipogenic and osteogenic lineages when maintained in the appropriate differentiation media. The differentiation was characterized by Oil red O and Alizarin red staining as shown in Figure 4.4. Enhanced mineralization was observed in ADSC-GMs as compared to 2D cultures on staining with Alizarin red (Figure 4.4c and d). ADSCs tend to mineralize the GMs during the process of osteogenic differentiation which will be advantageous when ADSC-GMs are used as *in vivo* delivery vehicles for bone regeneration. To further confirm and quantify

the differentiation, qPCR was performed to test the gene expression of respective differentiation markers, PPAR-γ for adipogenic and Runx2 for osteogenic differentiation. Markers for both lineages have shown significantly higher gene expression in ADSC-GMs as compared to ADSCs cultured in 2D by around 1.7 times and 1.8 times for adipogenic and osteogenic markers respectively (Figure 4.4e and f). Several studies have previously reported that microsphere scaffolds can enhance adipogenic and osteogenic differentiation of various kinds of stem cells (Park et al. 2010b, Moshaverinia et al. 2012, Yao et al. 2012). In agreement with those studies, our results also show that GMs also seem to be better platforms than 2D cultures for *in vitro* differentiation of ADSCs into adipogenic and osteogenic lineages.

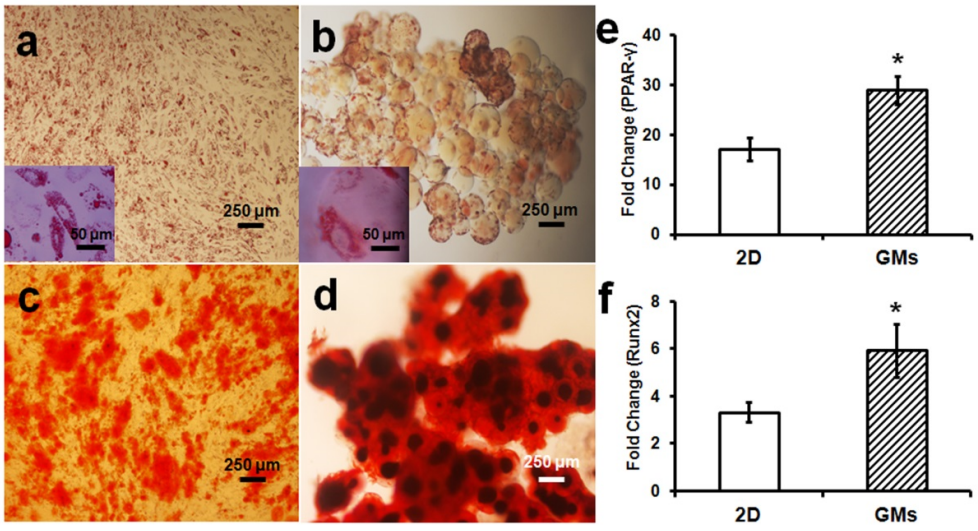

Figure 4.4 Optical microscope images of Oil Red O staining of ADSCs on (a) 2D and on (b) GMs showing adipogenic differentiation. Microscope images showing Alizarin red staining of ADSCs on (c) 2D and on (d) GMs for detection of osteogenic differentiation. qPCR fold change values measured relative to day 0 control for adipogenic and osteogenic marker genes (e) PPAR-γ and (f) Runx2 respectively on 2D and GMs. Error bars represent SD (n=3); *P<0.05 (student's t-test).

4.2.5. Hepatic differentiation of ADSCs

ADSCs were also differentiated towards the hepatic lineage and characterized using immunofluorescence staining and qPCR. Figure 4.5a and b show the images taken using CLSM after the differentiated ADSCs on 2D and on GMs were stained with fluorescent hepatic marker antibodies for albumin, alpha-fetoprotein and cytokeratin18. Phalloidin-TRITC and Hoechst were used to stain actin filaments and nucleus respectively to show the cell morphology. ADSCs showed positive immuno-staining for all three key hepatic markers both on 2D and on GMs. qPCR results (Figure 4.5c) show that albumin expression in ADSC-GMs is almost two times to that of in 2D cultures although it is not statistically significant. Most of the previous studies that show hepatic differentiation of ADSCs were all performed on conventional 2D cultures (Banas et al. 2007, Talens-Visconti et al. 2007, Coradeghini et al. 2010). Consistent with those studies, we were also able to differentiate ADSCs into hepatic lineage on 2D. However, such hepatocytes generated on 2D cannot be used directly for *in vivo* applications. On the other hand, our results show that GMs can also support the hepatogenesis of ADSCs. Such hepatic constructs derived from ADSC-GMs can be used in developing organoid grafts for liver tissue engineering.

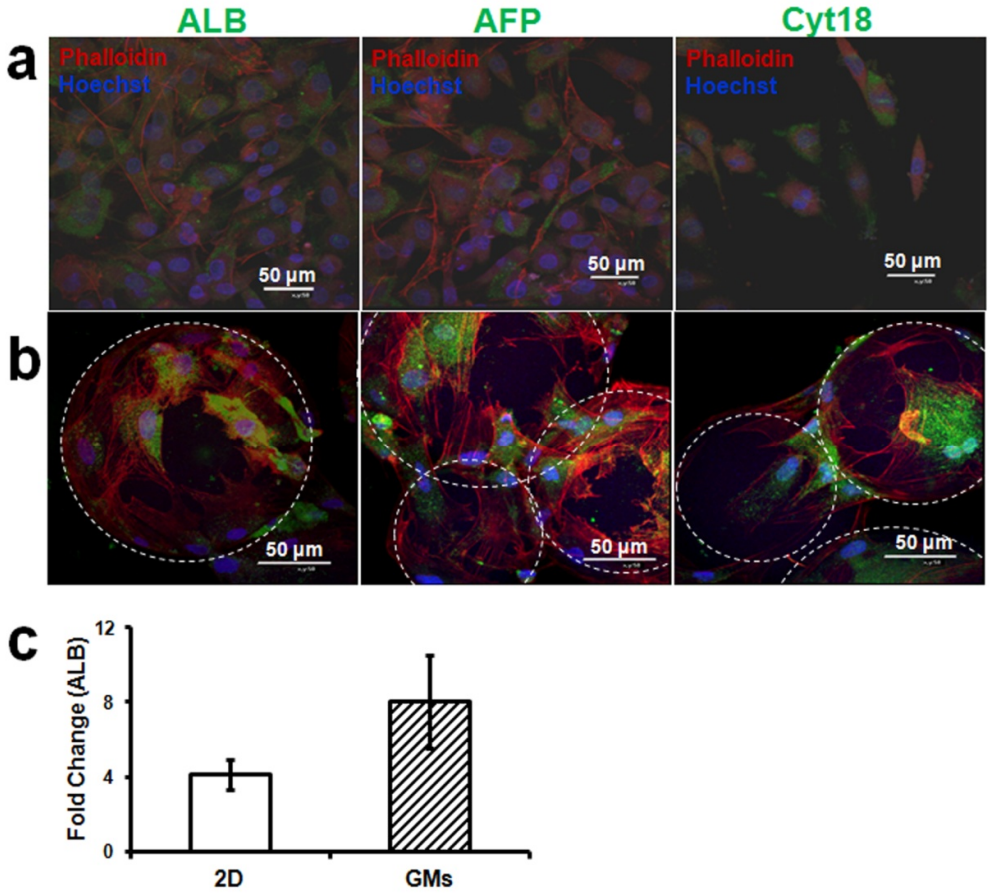

Figure 4.5 CLSM images of ADSCs differentiated towards hepatic lineage on (a) 2D and on (b) GMs after 2 weeks. For all CLSM images cell actin was stained with phalloidin-TRITC and nucleus with Hoechst. Hepatic markers were stained with respective antibodies tagged with FITC (albumin (ALB), alpha-fetoprotein (AFP) and cytokeratin 18 (Cyt18)). The dotted circles show the microspheres. (c) qPCR fold change values of ADSCs differentiated on 2D and GMs measured relative to day 0 control for hepatic marker gene albumin. The differences in expression levels were not found to be statistically significant. Error bars represent SD (n=3).

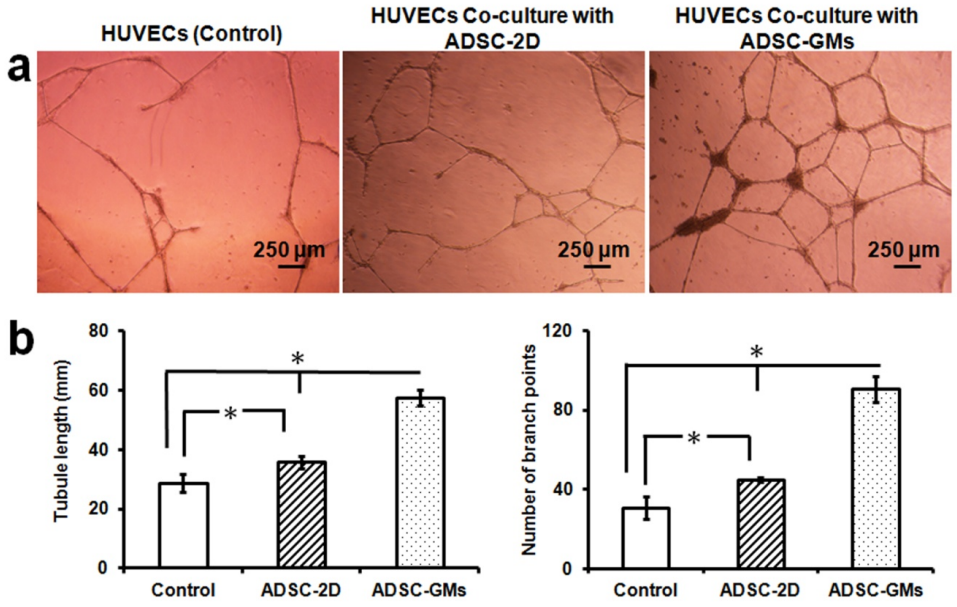

Figure 4.6 (a) HUVEC tube formation in two dimensional matrigel assay. Representative images of HUVECs seeded on matrigel in co-culture with or without ADSC-2D or ADSC-GMs. (b) Quantification of tube like formations. Tube lengths and number of branch points were estimated from images taken from three experiments. Error bars represent SD. *P<0.05. ANOVA followed by Tukey-Kramer test was performed to find out statistical significance.

4.2.6. Pro-angiogenic activity of ADSC-GMs

Angiogenic activity of ADSCs cultured on 2D and on GMs was studied using two dimensional *in vitro* matrigel assay which is a highly specific assay for angiogenesis and is widely used as an *in vitro* method to evaluate pro-angiogenic factors (Auerbach et al. 2003, Ucuzian et al. 2007). HUVECs on matrigel were co-cultured either with ADSCs on 2D or with ADSC-GMs for 24 h and optical microscope images of HUVEC tubules formed were taken. Figure 4.6a shows the representative images of HUVEC tubules formed on matrigel in different culture samples. Angiogenic activity was further quantified by measuring the tube lengths and the number of branch points of HUVEC tubular networks. As shown in Figure 4.6b, ADSC-GMs were able to induce 1.6 times longer tubules with 2 times more number of branch points as compared to ADSCs cultured on 2D. This shows the enhanced pro-angiogenic ability of ADSC-GMs compared to 2D

cultures. HUVECs alone without any co-culture with ADSCs were used as control for this study. Tubular formation of HUVECs in case of both the co-culture samples i.e., either with ADSC-GMs or with ADSCs on 2D were significantly higher compared to the control, inducing 2 times and 1.2 times longer tubules with 3 times and 1.5 times more number of branch points respectively. This highlights the inherent pro-angiogenic properties of the ADSCs. Similar enhancement in the tubular formation ability of HUVECs on matrigel was also reported earlier when co-cultured with human MSCs immobilized within RGD-grafted alginate microspheres (Bidarra et al. 2010).

4.3. Discussion

In this study we demonstrated a strategy to assemble the ADSCs and GMs into cell-microsphere constructs which seem to have enhanced regenerative properties compared to ADSCs cultured on 2D. Stem cell properties such as proliferation, maintenance of stemness, differentiation and pro-angiogenic abilities play crucial role in different stages of tissue regenerative process and platforms that can enhance such properties will aid in accelerating the wound healing. Designing such regenerative systems using injectable delivery vehicles such as GMs will make them a favourable option for clinical practice than the implant systems that need more invasive surgical means. Injectability of similar cell-microsphere construct systems into animal models using a syringe has been demonstrated in previous studies (Chung et al. 2009, Woo et al. 2014). For example, Chung et al. have used 18G needles to inject their 3T3 L1 mouse preadipocyte cellular aggregates made using microsphere scaffolds for adipose tissue regeneration in mice (Chung et al. 2009).

GMs have been employed in several previous studies for different tissue engineering applications such as cartilage tissue engineering (Garcia Cruz et al. 2013), muscle regeneration (Hagiwara et al. 2013), bone tissue engineering (Tzouanas et al. 2014) and also for culturing HUVEC cells (Zhu et al. 2008) both under *in vitro* and *in vivo* conditions and were found to be very advantageous

scaffolds in terms of eliciting biological response as well as in regeneration of tissues. GMs are also being employed to provide biomolecular cues by releasing appropriate growth factors at desirable rates in a controlled fashion (Park et al. 2005, Patel et al. 2008) and also as cell delivery vehicles (Lau et al. 2010). In addition, GMs are incorporated into cell aggregates (Bratt-Leal et al. 2011, Hayashi et al. 2011, Baraniak et al. 2012, Bratt-Leal et al. 2013) and cell sheets (Solorio et al. 2012b) to enhance the biological functions of respective systems. GMs are also widely studied by Prof Tabata's group for several drug delivery and tissue engineering applications (Kimura et al. 2003, Ogawa et al. 2010, Nakaguchi et al. 2012, Ikeda et al. 2014) including clinical trials (Nitta et al. 2009, Toyama et al. 2012) and thus regenerative systems made from them can have a better chance to succeed at clinical level compared to many other commercially available microcarriers.

In addition to the advantages discussed above, GMs fabricated using the water-in-oil emulsion technique also seem to be hydrogel in nature and have a tendency to swell by absorbing the liquid in which they are immersed. This swelling nature of GMs is very advantageous for encapsulating growth factors. Growth factors having opposite charge to that of the gelatin can be easily encapsulated using the principle of poly-ion complexation, just by dropping a small amount of growth factor solution onto the GMs (Young et al. 2005). The GMs will absorb the liquid completely during swelling and thus can have an almost quantitative encapsulation of the growth factor (Ikada et al. 1998). This permits GMs to be also used for spatio-temporal presentation of appropriate biomolecular signals in a controlled manner to influence stem cell fate (Solorio et al. 2012a).

Previous studies indicate the low survival rate of stem cells upon direct injection of cell suspensions to the injured tissue, possibly due to lack of cell-matrix interactions leading to anoikis (Terrovitis et al. 2010). On the other hand, delivering stem cells with three dimensional platforms have been able to improve the survival rate of the stem cells and thus further improved their tissue regeneration ability (Zakharova et al. 2010, Parisi-Amon et al. 2013). In a similar effort, here we developed the 3D ADSC-GM constructs which can deliver

ADSCs with enhanced regenerative properties. In addition to the differentiation studies which are most commonly performed, we also studied the effect of such 3D GM delivery vehicles on the stemness and angiogenic properties of ADSCs which are not well studied for such stem cell delivery constructs.

Maintaining the stemness properties of ADSCs during their propagation is crucial for keeping their multi-lineage differentiation properties intact, especially when the final goal is to utilize them for tissue regenerative applications. Although, the factors associated with stemness maintenance in embryonic stem cells are relatively well studied, those that influence stemness in adult stem cells such as ADSCs are still unclear (Leong et al. 2012). In the case of embryonic stem cells, it is well known that transcription factors such as Oct4, Sox2 and Nanog play a key role in maintaining their pluripotency. However, recent studies also indicate the expression of these pluripotent marker genes in ADSCs (Baer et al. 2010, Cheng et al. 2012). Very recently, Heneidi et al. have isolated a new set of multi-lineage differentiating cell population from adipose tissue which expresses many pluripotent marker genes (Heneidi et al. 2013). These studies highlight the growing interest towards identifying adult stem cells with pluripotent capabilities and for platforms which can enhance such properties. However, previous studies indicated the loss of stemness properties of MSCs when cultured in traditional 2D cultures (Park et al. 2010a) as also indicated by the downregulation of stemness marker gene expression of Oct4, Sox2 and Nanog in this work. This might lead to spontaneous differentiation of ADSCs into unwanted lineages (Tsai et al. 2010) and thus might significantly reduce the number of undifferentiated multipotent stem cells available for tissue regeneration. ADSC-GMs can overcome that problem by providing an artificial stem cell niche which preserves the expression of stemness genes.

ADSCs ability to differentiate into adipogenic and osteogenic lineages has been well-established both on 2D (Bunnell et al. 2008b) and on 3D scaffolds (Li et al. 2005). Utilizing such differentiated cells combined with various 3D scaffolds both for fat and bone tissue regeneration have also shown promising results (Chung et al. 2011, Hwang et al. 2013b, Zuk 2013). Previous studies have also employed

various kinds of microsphere scaffolds for MSC differentiation into adipogenic and osteogenic lineages. For example, PLGA microspheres coated with RGD peptide and loaded with BMP2 were used for osteogenic differentiation of human bone marrow MSCs (Park et al. 2010b). PLGA microspheres need to be coated with cell adhesive peptides to support cell adhesion and the growth factors need to be usually added during the fabrication of the microspheres which can significantly affect their biological activity. In another study, alginate microspheres were employed to encapsulate different kinds of stem cells and studied their adipogenic and osteogenic differentiation abilities (Moshaverinia et al. 2012). However, the alginate microspheres in this study were not modified with cell adhesion ligands, which affected the cell viabilities. Yao et al. has developed another method using a non-contact high voltage dispersion microsphere generating device to fabricate alginate-collagen microspheres, which showed enhanced adipogenic differentiation compared to 2D (Yao et al. 2012). Compared to other microsphere systems, GMs can support cell adhesion without any additional coating, easy to fabricate and even the growth factor encapsulation can be done much easily after the fabrication of microspheres without effecting their biological activity.

Hepatic differentiation of ADSCs especially in 3D scaffolds has been relatively less studied. Although there are many studies which show hepatic differentiation of ADSCs using different inducing agents (Banas et al. 2007, Talens-Visconti et al. 2007, Coradeghini et al. 2010), almost all of these studies were performed on 2D with very few focusing on 3D scaffolds (Wang et al. 2010). In this study, along with adipogenic and osteogenic differentiation of ADSCs, we have also shown differentiation of ADSCs to hepatic lineage both on 2D and on GMs. This allows ADSC-GM constructs to be used in forming organoid grafts for liver tissue engineering applications also along with being suitable for fat and bone regeneration.

Along with replenishing the lost cells during tissue repair, stem cells can also play a significant role in vascularisation of the regenerating tissues through paracrine signalling (Hoch et al. 2012). Previous studies have shown that ADSCs can

influence angiogenesis *in vivo* (Matsuda et al. 2013) and can also enhance tube formation ability of endothelial cells *in vitro* (Merfeld-Clauss et al. 2010). ADSCs are known to secrete several growth factors at bioactive levels such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and cytokines such as granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Salgado et al. 2010, Casadei et al. 2012) which have pro-angiogenic properties and have been reported to enhance HUVEC tubule formation under *in vitro* conditions (Lee et al. 2005, Chung et al. 2010, Botto et al. 2011). It has also been previously reported that the secretion profiles of such pro-angiogenic agents from ADSCs can be modulated by external conditions such as hypoxia (Rubina et al. 2009) or by culturing the ADSCs in 3D culture systems (Liu et al. 2011). The enhanced formation of HUVEC tubular networks observed in our study when co-cultured with ADSC-GMs compared to ADSC 2D cultures could be probably due to enhanced secretion of any of the above mentioned pro-angiogenic growth factors or cytokines under 3D conditions. Hence, the results obtained in our study suggest that angiogenic response of ADSCs can also be modulated through 3D culture conditions such as ADSC-GM constructs.

Overall, this kind of a biomaterials approach of ADSC-GMs for treating damaged tissues can be an effective approach which can aid the ADSCs to differentiate into a specific lineage that helps in regaining the functional properties of the tissue as well as in improving the vascularisation which further helps in the proper supply of nutrients all over the tissue. In addition, future studies can focus on modifications in chemical and mechanical properties along with encapsulation of growth factors into GMs which might be able to further modulate the ADSCs response and can aid in designing ADSC-GMs for various specific tissue engineering applications.

4.4. Conclusions

This study demonstrates a simple strategy to form cell-microsphere (ADSC-GMs) constructs with strong cell-cell and cell-matrix interactions. Our findings show that this assembly of ADSC-GMs can preserve the stemness properties and enhance the differentiation abilities of ADSCs into adipogenic, osteogenic and hepatic lineages when compared to the traditional 2D cultures. Moreover, the ADSC-GMs also enhance the tubular network formation of HUVECs on matrigel and thus seem to augment the pro-angiogenic properties of ADSCs. This would lead to better vascularization of the regenerating tissue. We believe that employing these ADSC-GMs with such enhanced regenerative abilities can accelerate tissue regeneration and thus enhances the therapeutic potential of ADSCs.

CHAPTER 5

OSTEOGENIC INDUCTION OF HUMAN ADIPOSE DERIVED STEM CELLS IN A COLLAGEN HYDROGEL – GELATIN MICROSPHERE COMPOSITE SCAFFOLD

This chapter describes the strategy of hydrogel-microsphere (Col-GMs) composite scaffold for ADSC delivery. In addition, regulation of ADSC behaviour using mechanical and biomolecular cues provided by the Col-GM scaffolds with osteogenic model as a focus will also be presented in this chapter. The work in this chapter addresses objectives 2 and 3 described in chapter 1.

5.1. Introduction

Three dimensional (3D) scaffolds made of biomaterials have been an integral part of the tissue engineering strategies. They act as temporary replacements for natural extra-cellular matrices (ECM) found *in vivo* and also play a key role in providing the appropriate biochemical and biomechanical cues for cells *in vitro*. Controlling cell behaviour using these 3D scaffolds has become a major area of focus in tissue engineering, for which a wide variety of scaffolds have emerged. Among them include, two widely used scaffolds that are microspheres and hydrogels. In chapter 4, the study of cell-microsphere constructs (ADSC-GMs) has shown the influence that gelatin microsphere (GM) scaffolds can have on the tissue regenerative properties of ADSCs by enhancing their stemness, differentiation and angiogenic properties. However, maintaining such microsphere scaffolds at the injury site has been found to be challenging (Pannek et al. 2001, Lemperle et al. 2004) and in turn cell-cell and cell-matrix interactions present in the cell-microsphere constructs that are crucial for tissue integration can also get destroyed during the delivery process (Chen et al. 2011). To overcome such challenges associated with ADSC-GM constructs, we aimed to fabricate a hydrogel-microsphere composite scaffold system (Col-GMs) by encapsulating the ADSC-GM constructs into collagen hydrogels (Col). The encapsulating collagen hydrogel of the Col-GM system will enable gelling of the GMs together thereby restricting the ADSC-GMs to the injury site and also keeping the cell-cell interactions intact. Such a composite scaffold can therefore aid in making the ADSC-GMs suitable for injectable delivery as well as in the regulation of ADSC behaviour. In this work, we have chosen osteogenic induction of ADSCs as a model study, to establish the capability of Col-GM scaffolds in regulating the ADSC behaviour through presentation of various mechanical and biomolecular cues.

As Col-GM scaffolds contain both the microspheres and hydrogel, the behaviour of encapsulated ADSCs will be governed by the properties of both the individual scaffolds. While microspheres are widely popular as carriers which can deliver both cells and growth factors simultaneously (Chen et al. 2010), hydrogels are

more known for providing a 3D *in vivo* mimicking environment (Slaughter et al. 2009). In addition, the stiffness of a hydrogel environment also tends to play a role in determining stem cell fate with stiffer matrices enhancing the osteogenic differentiation (Engler et al. 2006). The Col-GM composite scaffolds that we intend to develop will be able to present hydrogel stiffness effect as mechanical cues to drive the ADSCs towards osteogenic lineage. In addition, to further accentuate the osteogenic induction, we also provided biomolecular cues to the ADSCs encapsulated in Col-GM scaffolds through basic fibroblast growth factor (bFGF) controlled release from GMs. bFGF is an important growth factor which regulates multiple regenerative processes simultaneously, such as cell proliferation, migration, angiogenesis and wound healing (Lee et al. 2002, Miyoshi et al. 2005, Hosseinkhani et al. 2006, Schmidt et al. 2006). In addition, many studies have also highlighted the potential of bFGF to enhance the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) (Pitaru et al. 1993, Hanada et al. 1997, Oh et al. 2012) and in turn for bone regeneration (Jiang et al. 2010, Omata et al. 2012).

In order to provide the mechanical cues through collagen hydrogels, we employed an unconventional approach i.e., variation in the matrix rigidity has been brought in by incorporating different amounts of GMs. Traditional crosslinking methods of enhancing hydrogel strength are usually cytotoxic and do not support *in situ* cell encapsulation. Alternatively, incorporating a harder phase such as microparticles into a hydrogel has been shown to enhance the overall rigidity of the composite scaffold acting as reinforcements for softer matrices (Jha et al. 2009, Hu et al. 2011a).

Thus, in this study, we investigated the effect of varying GMs concentration in reinforcing the collagen hydrogels and performed rheological studies on the bulk rigidity of the hydrogel. In addition, we also studied the effects of the mechanical cues on the osteogenic differentiation ability of the ADSCs. Differentiation was characterized by studying the gene expression of osteogenic marker genes bone morphogenetic protein 2 (BMP2), osteocalcin (OCN) and Runx2 and also by quantifying the alkaline phosphatase (ALP) activity. Further, we encapsulated

bFGF in the Col-GM scaffolds and also studied the effect of bFGF controlled release on the osteogenic differentiation of ADSCs and characterized as above. Overall, this study shows that Col-GMs can be used as an efficient delivery system for ADSCs as well as necessary growth factors simultaneously, with the capability to drive ADSCs towards osteogenic lineage through the means of mechanical and biomolecular cues.

5.2. Results

5.2.1. Characterization of mechanical properties of Col-GMs

Dynamic mechanical analysis was performed using an AR-G2 rheometer to study the effect of incorporation of GMs on the mechanical properties of the collagen hydrogels. Two varieties of Col-GM scaffolds were prepared by incorporating varying amounts of GMs into the hydrogel – Col-10-GMs (10 mg of GMs per 1 ml of collagen gel) and Col-20-GMs (20 mg of GMs per 1 ml of collagen gel). Strain sweep tests were conducted to find out the linear visco-elastic region (Figure 5.1). Later, frequency sweep studies (Figure 5.2a, b and c) of different scaffolds were performed by fixing the strain amplitude at 1%. Dynamic storage moduli (G') of all the three scaffolds was found to be greater than their respective loss moduli (G") over the entire frequency range studied (0.1 – 10 rad/s). This indicates all the three scaffolds are showing an elastic solid like behaviour (Liu et al. 2013). In addition, G' of all the scaffolds kept increasing with the increase in frequency which is indicative of the reduced network structures relaxation at higher frequencies (Zhou et al. 2011). Also, the G' values of all three scaffolds at a frequency of 1 rad/s were compared to assess their relative gel strengths as shown in Figure 5.2d. G' of Col-20-GMs was found to be significantly higher compared to that of Col and Col-10-GMs by about 32 times and 5 times respectively. Another way to assess the gel strengths is by comparing their tan δ values which is the ratio of G" to that of G' (Celli et al. 2007). A value of tan $\delta \leq 1$, is indicative of a gel like material and lower the value of tan δ more elastic is the material, while tan $\delta \geq 1$ indicates a sol state. Figure 5.2e shows that tan δ of

all scaffolds is less than 1, confirming the gelation and also the values are significantly different from each other for all the scaffolds with Col-20-GMs being the stronger gel of all.

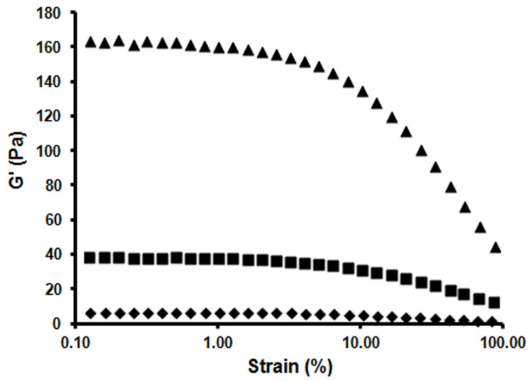

Figure 5.1 Strain sweep study to identify the linear visco-elastic region showing G' (storage modulus) values of collagen hydrogel (◆), Col-10-GMs (■) and Col-20-GMs (▲).

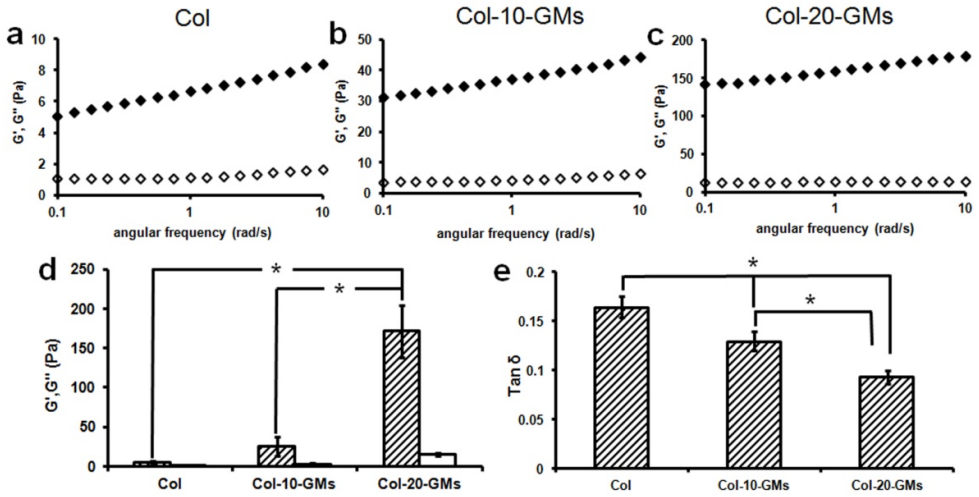

Figure 5.2 Rheological properties of Col-GM scaffolds. G' (◆) – storage modulus and G" (◇) – loss modulus of (a) collagen hydrogel (b) Col-10-GMs (collagen hydrogel containing 10 mg of GMs) and (c) Col-20-GMs (collagen hydrogel containing 20 mg of GMs). (d) G' (▨) and G" (□) of replicate samples measured at a strain amplitude of 1% and an angular frequency of 1 rad/s. (e) Tan δ values of different scaffolds. G' and tan δ values indicating Col-20-GMs having higher gel strength compared to Col-10-GMs and Col. Error bars represent SD (n=3); *P<0.05 (student's t-test).

5.2.2. ADSC culture in Col-GM scaffolds

ADSCs were cultured in Col-GM scaffolds over a period of 10 days. Optical microscope (Figure 5.3a) and CLSM images (Figure 5.3b) were taken on various time points during the culture to study the morphology and migratory behaviour of ADSCs. ADSCs were stained with Hoechst for nucleus (blue) and Phalloidin-TRITC (red) for actin filaments before CLSM imaging. GMs being hydrogel in nature seem to absorb both the stains and could not be completely washed off, but clear distinction can still be made of the stained ADSCs from the GMs in the images shown in Figure 5.3b. As the ADSCs were initially seeded on the GMs and then loaded into the collagen hydrogels, on day 1 most of ADSCs seem to be adherent to the GMs. Cells started migrating into the surrounding gel space during day 4 to day 7 and found to completely populate the scaffold by day 10, covering both the GMs and the surrounding gel space. Previous work done by Chen et al. with a similar microsphere-hydrogel hybrid scaffold for neural tissue engineering has shown that this type of a composite scaffold can also aid in maintaining the cell-cell and cell-matrix interactions (Chen et al. 2011). Such interactions are prominent in the cell-microsphere scaffolds which were found to be crucial for maintaining the enhanced biological functions of the cells (Zhu et al. 2007a, Zhu et al. 2007b, Chen et al. 2012) and need to be preserved during cell delivery applications. With this motivation, in this study, we devised Col-GM scaffolds for ADSC culture. However, in slight contrast to the previous work with neuronal cells in the microsphere-hydrogel hybrid scaffolds, ADSCs have shown much more of a migratory behaviour into the surrounding gel space and populated the whole scaffold. As collagen is one of the most abundant proteins in the ECM of many of the human tissues, this phenomenon of ADSC migration into the surrounding collagen gel matrix seems to mimic the stem cell integration with the host tissue. This scaffold system can thus be effectively used to propagate the ADSCs for expanding their cell numbers and then to deliver those stem cells in an appropriate matrix for repairing damaged tissues.

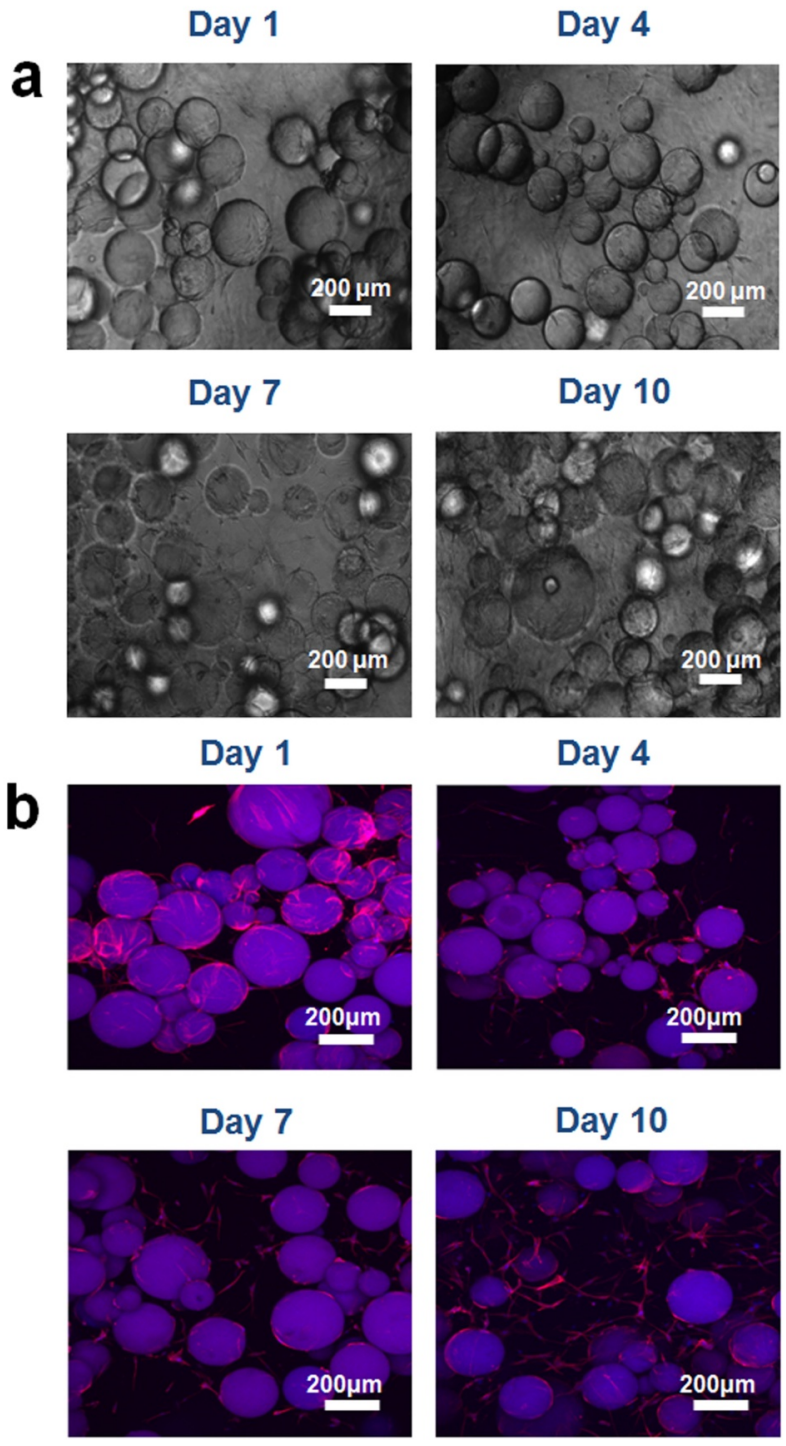

Figure 5.3 (a) Optical microscope and (b) Confocal laser scanning microscope (CLSM) images of human ADSCs cultured in Col-20-GM scaffolds over 10 days of culture showing cell adhesion and migratory behaviour. For CLSM images cell actin was stained with phalloidin-TRITC and nucleus with hoechst.

5.2.3. Osteogenic differentiation of ADSCs in Col-GM scaffolds

ADSCs cultured in the Col-GM scaffolds were tested for their osteogenic differentiation ability upon maintaining in the differentiation media. The differentiation was characterized by studying the gene expression of osteogenic marker genes – BMP2, OCN and Runx2 (Figure 5.4) and ALP activity (Figure 5.5). Gene expression of OCN and Runx2 was studied on day 7 and day 14. While for BMP2, as our preliminary experiments showed higher amounts of it being expressed in the initial stages of differentiation rather than later stages, we also studied their expression on day 4 along with on day 7 and day 14. BMP2 expression seems to be highest on day 4 and tend to reduce by day 7. By day 14, BMP2 expression levels tend to reduce further or remain similar to day 7 in different scaffolds. For Runx2 the expression levels remained almost similar on day 7 and day 14 for all scaffolds and in case of OCN, which is a late marker gene, the expression levels have increased from day 7 to day 14 varying from 1.2 to 1.9 times in different scaffolds. For all three genes that we studied, on all the time points, mostly both Col-10-GMs and Col-20-GMs seem to have similar expression pattern but have shown significantly increased expression compared to pure collagen hydrogels. Also, ADSCs in Col-10-GMs and Col-20-GMs have shown significantly higher expression of BMP2 and OCN on all the time points as compared to ADSCs cultured on GMs. In addition to the gene expression studies, ALP activity was also measured of the Col-GM scaffolds on day 7 and day 14 (Figure 5.5). ALP activities in all the scaffolds found to be increasing from day 7 to day 14. On both the time points, ALP activities were significantly higher in the Col-10-GM and Col-20-GM scaffolds compared to the Col gel by about 3.1 and 6.4 times respectively. Further ADSCs differentiated in Col-20-GMs have shown twice the amount of ALP activity compared to the Col-10-GM samples. Overall, these results show that ADSCs have better osteogenic differentiation abilities in Col-GM composite scaffolds compared to either pure collagen hydrogels or GMs alone.

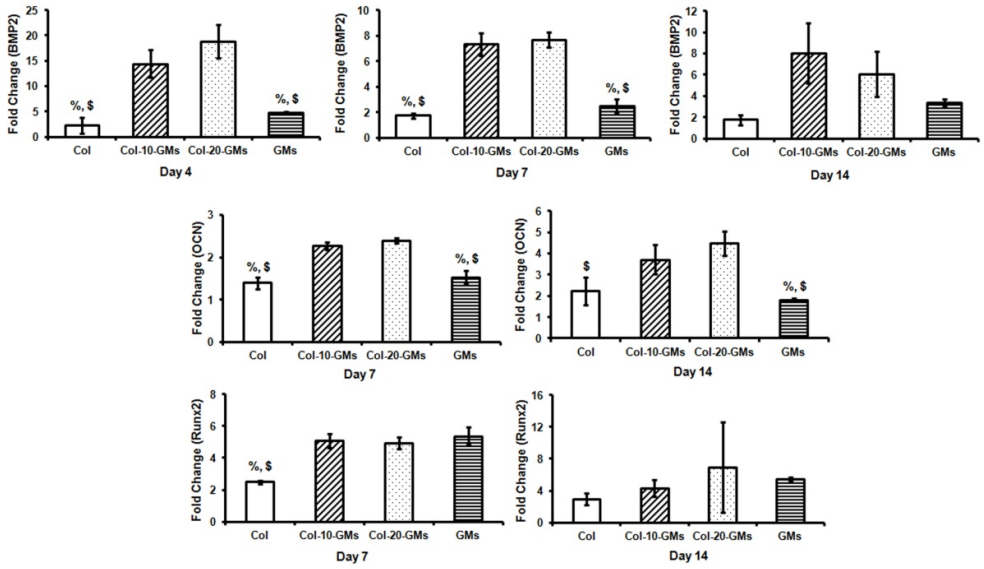

Figure 5.4 qPCR fold change values of osteogenic marker genes BMP2, OCN and Runx2 upon differentiating with osteogenic induction media in various scaffolds, measured relative to day 0 controls. β-actin used as housekeeping gene. Error bars represent SD (n=3); % and $ represents P<0.05 (student's t-test) analyzed with respect to Col-10-GMs and Col-20-GMs respectively.

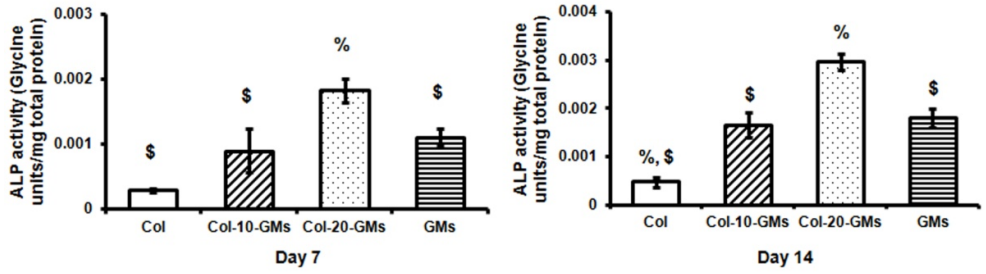

Figure 5.5 ALP activity values of ADSCs upon differentiating with osteogenic induction media in various scaffolds. Glycine unit can be defined as the amount of enzyme causing the hydrolysis of 1 µmol of p-nitrophenyl phosphate per minute at pH 9.8 and 25 ^{o}C (glycine buffer). Error bars represent SD (n=3); % and $ represents P<0.05 (student's t-test) analyzed with respect to Col-10-GMs and Col-20-GMs.

5.2.4. bFGF encapsulation and *in vitro* release from Col-GM scaffolds

GMs are well established drug delivery vehicles and have been used on numerous occasions to controlled release biomolecules for enhancing cellular activity (Kawai et al. 2000, Zhu et al. 2008, Jin et al. 2011, Hagiwara et al. 2013). In this study, we aim to use GMs as growth factor depots in Col-GM scaffolds to provide the ADSCs with necessary biomolecular cues in a controlled fashion. To achieve this objective, we first encapsulated bFGF into three scaffolds – Col, Col-20-GMs and GMs and studied its release profile (Figure 5.6) under *in vitro* conditions over a period of 14 days. The burst release which is an undesirable trait usually associated with controlled release of encapsulated growth factors from delivery systems (Joung et al. 2008) was found to be very minimal in all the three scaffolds that we studied releasing only 5.3%, 4.7% and 3.7% of the encapsulated bFGF in the first two days from Col, GMs and Col-20-GM scaffolds respectively. After 14 days of incubation, the scaffolds have released around 22.3%, 16.9% and 12.8% of the encapsulated 100 ng of bFGF from Col, GMs and Col-20-GM scaffolds respectively. The amount of bFGF released by GMs over 14 days seems to be consistent with another study conducted previously (Zhu et al. 2008).

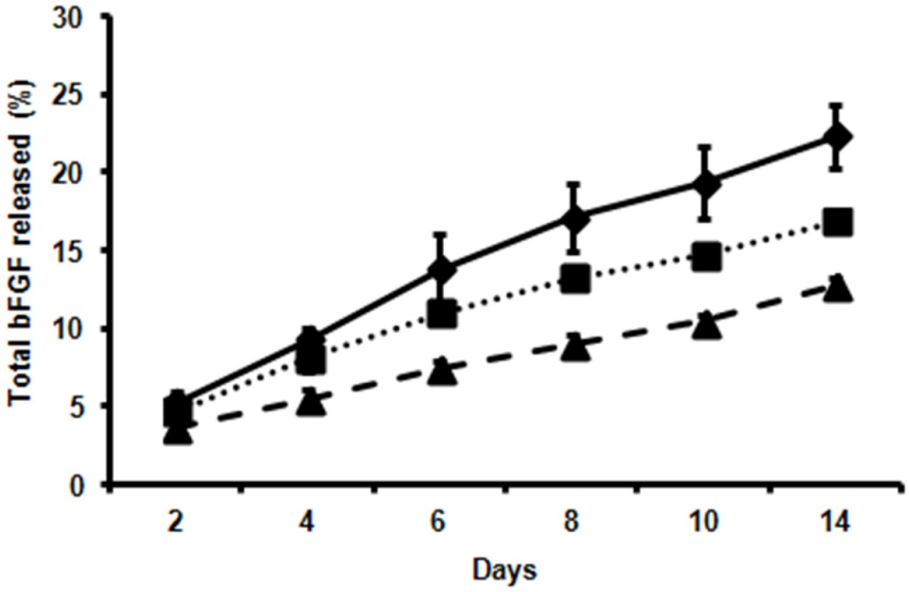

Figure 5.6 *In vitro* release profiles of bFGF from different scaffolds over a period of 14 days. —◆— Col, ···■·· GMs and – – ▲ – – Col-20-GMs.
Error bars represent SD (n=3). Differences between the total bFGF released from all three scaffolds at each time point were found to be statistically significant, $P<0.05$ (one-way ANOVA).

5.2.5. Effect of bFGF controlled release on osteogenic differentiation of ADSCs in Col-GM scaffolds

bFGF is a very well studied growth factor and known for its role in cell proliferation, angiogenesis and osteogenesis (Pitaru et al. 1993, Hosseinkhani et al. 2006, Schmidt et al. 2006). As there is no significant difference in the osteogenic marker gene expressions upon differentiation between Col-10-GMs and Col-20-GMs (Figure 5.4) and Col-20-GMs having shown higher ALP activity (Figure 5.5), we have chosen only the later scaffold for studying the effect of bFGF controlled release on osteogenic differentiation of ADSCs. In order to study this, bFGF was first encapsulated in Col, Col-20-GMs and GM scaffolds and ADSCs cultured in those scaffolds were induced towards osteogenic lineage. The differentiation was characterized by studying the gene expression of osteogenic marker genes OCN, Runx2 on day 7 and day 14 and that of BMP2 on day 4 along

with day 7 and day 14 (Figure 5.7). A set of samples with free bFGF provided in the medium were used as control group.

Our results show that there is high amount of BMP2 gene expression during the early phase of differentiation. For example, on day 4, average fold change values of BMP2 gene expression in both Col-20-GMs and GMs with encapsulated bFGF were found to be 62.3 and 27.3 with both of them being about 2.3 times higher than their respective control groups. By day 7, there was a reduction of expression varying from 2 to 6 times in different scaffolds which was further decreased by day 14. For OCN, as it is a late marker, there was no significant difference in the expression levels among the different scaffolds on day 7. By day 14, OCN expression was found to be higher in Col, Col-20-GMs and GMs encapsulated with bFGF by about 1.4, 3.6 and 1.7 times compared to their respective control groups. bFGF loaded Col-20-GMs were found to be showing highest OCN expression of all the scaffolds. In the case of Runx2 expression, by day 14, all the three scaffold types have similar expression levels with bFGF loaded scaffolds inducing higher expression levels compared to their respective control groups. The gene expression fold change values obtained in our study were found to be comparable to several previous studies (Gaharwar et al. 2012, Oh et al. 2012, Wang et al. 2014, Zhang et al. 2014). ALP activity values on both time points day 7 and day 14, also show a similar trend to that of gene expression, with bFGF loaded scaffolds showing higher activity compared to their control groups (Figure 5.8). ALP activities in all scaffolds found to be increased from day 7 to day 14. In addition, bFGF loaded Col-20-GMs have shown highest ALP activity compared to all other scaffolds. Overall, our gene expression studies for the three osteogenic marker genes along with the ALP quantification assay results show that, Col-20-GMs encapsulated with bFGF tend to induce higher levels of osteogenic differentiation compared to all other scaffold types.

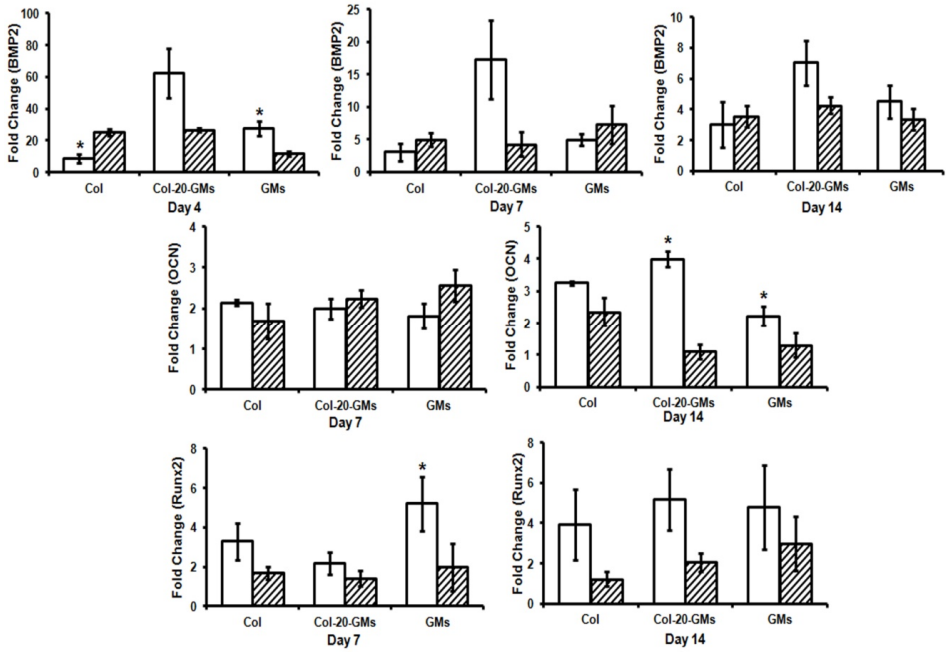

Figure 5.7 qPCR fold change values of osteogenic marker genes BMP2, OCN and Runx2 upon differentiating with osteogenic induction media in Col, Col-20-GM and GM scaffolds, measured relative to day 0 controls. β-actin used as housekeeping gene. ▭ bFGF encapsulated in the scaffolds and ▨ bFGF provided as a supplimentation in the media. Error bars represent SD (n=3); *P<0.05 (student's t-test) analysed between bFGF encapsulated samples with respect to bFGF as media supplementation samples.

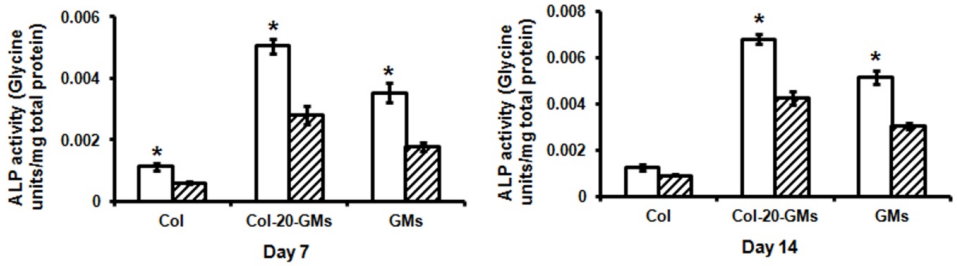

Figure 5.8 ALP activity values of ADSCs upon differentiating with osteogenic induction media in Col, Col-20-GM and GM scaffolds. Glycine unit can be defined as the amount of enzyme causing the hydrolysis of 1 µmol of p-nitrophenyl phosphate per minute at pH 9.8 and 25 ^{o}C (glycine buffer). ▭ bFGF encapsulated in the scaffolds and ▨ bFGF provided as a supplimentation in the media. Error bars represent SD (n=3); *P<0.05 (student's t-test) analysed between bFGF encapsulated samples with respect to bFGF as media supplementation samples.

5.2.6. Adipogenic differentiation in Col-GM scaffolds

In addition to osteogenic differentiation, we also wanted to study if the Col-GM scaffolds are also suitable for adipogenic differentiation of ADSCs. To this end, we induced the ADSCs encapsulated in pure collagen, Col-10-GMs, Col-20-GMs and GM scaffolds towards adipogenic lineage by culturing in adipogenic induction medium. We further characterized the differentiation by studying the gene expression of well known adipogenic marker gene, PPAR-γ using qPCR. In general, softer gels are known to promote adipogenic induction in stem cells as indicated by previous studies (Engler et al. 2006). In agreement with such studies, our qPCR results (Figure 5.9) also show that, higher levels of PPAR-γ was expressed in softer gels such as pure collagen hydrogels or Col-10-GMs compared to the relatively stiffer substrate like Col-20-GMs. Thus for fat specific applications, it is better to employ Col or Col-10-GM scaffolds rather than Col-20-GMs. Further studies involving appropriate growth factor controlled release, will be able to give more insights into tuning the Col-GM scaffold properties for adipose tissue based applications.

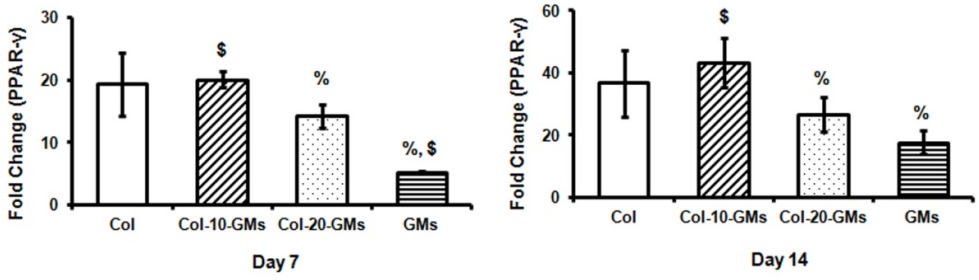

Figure 5.9 qPCR fold change values of adipogenic marker gene PPAR-γ upon differentiating with adipogenic induction media in various scaffolds, measured relative to day 0 controls. β-actin used as housekeeping gene. Error bars represent SD (n=3); % and $ represents $P<0.05$ (student's t-test) analyzed with respect to Col-10-GMs and Col-20-GMs respectively.

5.3. Discussion

This study describes a strategy to induce osteogenesis in human ADSCs using mechanical and biomolecular cues in a Col-GM composite scaffold. Col-GMs with varying hydrogel stiffness have been generated by varying the amount of GMs encapsulated which is employed as a mechanical cue. Further, the incorporated GMs were also used to controlled release bFGF. Along with providing a unique biomechanical microenvironment, such a composite scaffold can also overcome many disadvantages associated with the individual scaffolds as discussed in the introduction. However, most of the studies involving osteogenic induction of various stem cells have only been done either on microsphere or hydrogel scaffolds but very few studies (Wang et al. 2009a, DeVolder et al. 2012) focused on a microsphere-hydrogel composite scaffold system. Microsphere-hydrogel scaffolds have also been employed previously for other applications such as cartilage repair (Sukarto et al. 2012) and drug delivery through blood-brain barrier (Caicco et al. 2013). In this study, ADSCs encapsulated in the Col-GM scaffolds were able to show enhanced osteogenic differentiation with increase in scaffold rigidity and have shown much more induction upon bFGF controlled release.

GMs are well established drug delivery vehicles (Young et al. 2005) and also have been studied for many tissue engineering applications (Zhu et al. 2008, Baraniak et al. 2012, Leong et al. 2013). They have also been employed for various clinical trials successfully (Nitta et al. 2009, Toyama et al. 2012). Similarly, collagen hydrogels are also widely studied for tissue engineering applications and also many collagen based tissue engineering products have been commercialized by various companies for clinical applications ranging from cosmetics to bone regeneration (Malafaya et al. 2007). We believe, fabricating regenerative systems using such clinically successful materials would help in faster translation of those systems to the clinical stage. Further the Col-GMs developed here can also be delivered by injectable means which will be appealing to the medical field.

The scaffold design of Col-GMs allowed us to try and vary the matrix rigidity by varying the amount of GMs incorporated. The G' values for all the three Col-GM scaffolds (Figure 5.2d) obtained through rheological studies, shows that incorporating GMs into collagen hydrogels enhances the strength of the hydrogels. Also, increasing the amount of GMs in the collagen hydrogel will further enhance the mechanical strength of the composite scaffold. These results were also supported by the tan δ data (Figure 5.2e). During deformation, GMs with proper homogenous dispersion in the hydrogel will aid in effective transfer of the load from polymer chains and thus might be responsible for enhancing the mechanical strength of the hydrogel. These results, in accordance with other studies (Jha et al. 2009, Hu et al. 2011a), confirm the strategy of enhancing matrix rigidity by incorporating a relatively harder phase into the hydrogel. Moreover, GMs incorporated into collagen hydrogels also had a similar reinforcing effect to that of nanofillers such as silicate nanoparticles (Gaharwar et al. 2012), clay nanotubes (Liu et al. 2013) or chitosan nanofibers (Zhou et al. 2011) in other hydrogel systems. The G' values obtained for the Col-GMs were also similar to that obtained for a collagen hydrogel – PLGA microsphere composite system which was also employed for the osteogenic differentiation of mouse MSCs (DeVolder et al. 2012). The shear modulus (G) obtained in our study can also be mathematically converted into elastic modulus (E) in order to compare with the available literature of scaffolds employed for osteogenic differentiation. The mathematical relation between shear modulus and elastic modulus is given by $E = 2G(1+\gamma)$, where γ is the poisson's ratio. For the collagen hydrogel used in this study manufacturer (Advanced biomatrix) has suggested a poisson's ratio of 0.5 and the relation between E and G as $E = 3G$. As per this relation, the average elastic moduli of the Col-GMs scaffolds were found to be 15.9 Pa, 75.6 Pa and 513.8 Pa respectively for the collagen hydrogel, Col-10-GMs and Col-20-GMs. The typical scaffolds used for treating skeletal defects have the elastic modulus in the range of kilo to mega pascals (Hu et al. 2011b, Frohbergh et al. 2012, Tan et al. 2014). However, softer gels such as uncrosslinked collagen hydrogels have also been employed and were reported previously to be supportive of osteogenic differentiation (DeVolder et al. 2012, Oh et al. 2012). Such matrices can mimic the initial mesenchymal tissue conditions which initiate the osteogenic

differentiation of MSCs. In addition, although the initial rigidity of such softer gels is low, upon differentiation of MSCs for one week and further implantation in chicken chorioallantoic membranes (used as *in vivo* models for examining bone formation) the shear modulus values of the collagen hydrogel-PLGA microsphere composite scaffold constructs seem to significantly increase upto 35 kPa (DeVolder et al. 2012).

For characterizing the osteogenic differentiation of ADSCs in various scaffolds we have studied gene expression of well known osteogenic marker genes BMP2, OCN and Runx2 using qPCR and also ALP assay. All the three markers chosen were important for bone development and osteogenic differentiation during different stages. BMP2 is a widely studied cytokine in the context of bone development and is known to enhance the osteogenic differentiation by regulating the expression of alkaline phosphatase, type I collagen and Runx2 (Kaur et al. 2010). Runx2 is a transcription factor which plays a key role in the commitment of multipotent stem cells towards osteogenic lineage. It also acts as a positive regulator for the expression of bone matrix genes such as type I collagen, osteopontin, bone sialoprotein and Osteocalcin (Komori 2003). Previous studies employing Runx2 deficient mice have shown complete lack of bone and absence of osteoblasts in those mice, highlighting the important role Runx2 in osteogenic differentiation (Komori et al. 1997). Both BMP2 and Runx2 are early markers of differentiation and are expressed during the initial stages of differentiation. On the other hand, OCN is a late stage marker and expresses during the maturation stage of differentiation. Osteocalcin is a major noncollagenous protein present in the extracellular matrix of bone secreted by osteoblasts. It binds to hydroxyapatite and plays a key role in the matrix mineralization process. Other markers which are commonly employed to study osteogenic differentiation include osteopontin, type I collagen and bone sialoprotein. In addition, mineralization assays such as histochemical staining using alizarin red or von kossa stains are also used to assess the osteogenic differentiation of stem cells.

Along with gene expression studies we have also performed ALP assay to study the osteogenic differentiation. ALP plays a key role in the process of

mineralization during osteogenesis. ALP increases the local concentration of inorganic phosphate which is needed for hydroxyapatite formation and mineralization and decreases the concentration of extracellular pyrophosphate, which acts as an inhibitor of mineralization. Several tissue engineering based studies involving osteogenesis have employed ALP enzyme activity measurement or histochemical staining of ALP as a standard assay for assessing the success of differentiation as increased ALP activity is considered as a good predictor of mineralization (Golub et al. 2007, Anderson et al. 2011, Vines et al. 2012, Simoes et al. 2013, Choi et al. 2014, Maia et al. 2014).

The increase in the matrix rigidity of Col-GMs by GMs incorporation seems to favour the osteogenic differentiation of ADSCs. Previous studies have indicated the role of matrix elasticity in driving the stem cell differentiation towards osteogenic lineage (Engler et al. 2006). In this study, our gene expression and ALP assay results show that Col-20-GMs were able to induce more differentiation followed by Col-10-GMs and Col gels. This can be attributed to the mechanical properties of the scaffolds with stiffer ones inducing more differentiation. Our experiments of ADSCs cultured on GMs alone, presented in chapter 4, have shown that the differentiating ADSCs seem to mineralize the GMs (Figure 4.4d). This might probably aid in much better mineralization of the Col-GM scaffolds and in turn can enhance the osteogenic differentiation. Further, the stiffness effect of Col-GMs is also clearly evident as Col-20-GM scaffolds have shown higher amount of BMP2 and OCN gene expression along with higher ALP activity compared to GMs alone.

To further enhance the osteogenic differentiation of ADSCs in Col-GMs we then employed the biomolecular cues. It has been shown previously that encapsulating growth factors into GMs is much easier compared to other microsphere scaffolds (Young et al. 2005). It can be done by simply adding a very little amount of concentrated growth factor solution onto the dry microspheres. GMs during the process of swelling tend to absorb the liquid completely along with all the growth factor without any loss (Zhu et al. 2008). In our controlled release experiments, the burst release of bFGF was found to be minimal during the first two days.

Previous studies indicate that bFGF tends to form strong complexes with both collagen and gelatin which might inhibit the burst release significantly during the early phases (Kanematsu et al. 2004, Young et al. 2005). As GMs are crosslinked with glutaraldehyde, they seem to release the encapsulated bFGF at a slower rate compared to pure collagen gel which is uncrosslinked. In addition, GMs tend to encapsulate growth factors by forming strong ionic complexes (Young et al. 2005) allowing for the release to be more sustained. On the other hand, collagen used in this study has an isoelectric point in the zone of pH 7-8 (as informed by Advanced Biomatrix), which makes it neutral in charge at physiological pH and thus may not be able to form as strong ionic complexes with bFGF (isoelectric point of 9.6) as acidic gelatin (isoelectric point of 5.2) does in the case of GMs. This also leads to a relatively faster release of bFGF from collagen gels compared to GMs. However, the interactions of bFGF with collagen are not purely electrostatic alone and there might be other interactions which play a role but are not fully understood (Kanematsu et al. 2004). Such interactions might be responsible for having a sustained release from the Col hydrogels also, although the release is faster than the GMs. In the case of Col-20-GMs, the presence of a collagen gel layer surrounding the GMs as an additional barrier might be the cause for much slower release of encapsulated bFGF compared to either Col or GMs alone. However, none of the release profiles from any of the scaffolds have reached a plateau by the end of day 14 which suggests that these scaffolds can be used for long term controlled release applications.

Such a controlled release of bFGF from the Col-GMs was also found to induce higher osteogenic differentiation in the encapsulated ADSCs compared to the samples where in bFGF was supplemented in the media. This clearly highlights the importance of bFGF presentation through delivery vehicles in a controlled manner, compared to merely adding in the medium. Even among the controlled releasing samples, Col-20-GMs which have shown the slowest bFGF release rate have induced higher differentiation. Col-20-GMs because of their slower release rate were able to keep more bFGF within the scaffold for a longer time, nearer to the cell membranes of ADSCs. Such encapsulated bFGF might thus have a better chance to interact with the corresponding receptors on cell membranes and induce

higher levels of osteogenic signals. On the other hand, the bFGF released out from the scaffolds will be of use during the *in vivo* applications, to elicit favourable responses such as vascularization in the surrounding injured tissue. Also, ALP activities of encapsulated ADSCs presented with bFGF either by controlled release or by supplementation in the medium (Figure 5.8) were found to be higher than the samples without bFGF (Figure 5.5) ranging from 1.4 times to almost 4 times in different scaffolds. This signifies the pro-osteogenic ability of bFGF which is supported by many studies in the literature (Pitaru et al. 1993, Hanada et al. 1997, Rider et al. 2008, Oh et al. 2012).

Another interesting result obtained in this study is that bFGF controlled release was also able to induce high levels of BMP2 gene expression during initial stages of differentiation. Earlier studies using ectopic bone forming assays have indicated the importance of exposure to higher levels of BMP2 during initial stages for optimal bone formation (Bhakta et al. 2012). Such high levels of BMP2 expressed by ADSCs can thus act in autocrine manner and further help to enhance bone regeneration. In addition, many studies have also highlighted the importance of supplying both bFGF and BMP2 for enhancing bone regeneration (Hanada et al. 1997, Su et al. 2013, Wang et al. 2013a). On the contrary, our study shows that, releasing bFGF from Col-20-GMs in a controlled fashion will actually induce the ADSCs to express BMP2 by themselves, in higher amounts, which might avoid or reduce the need to supply exogenous BMP2. However, more studies need to be conducted to confirm the effect of such increased BMP2 expression on bone regeneration.

Overall, this study shows that within Col-GM scaffolds, behaviour of ADSCs can be modulated using a combination of biomechanical and biomolecular cues to drive them towards osteogenic lineage. In addition, as many studies have indicated previously, collagen of the Col-GM scaffolds can also be conjugated with hydroxyapatite or β-tricalcium phosphate to further enhance the osteogenic differentiation of ADSCs (Komaki et al. 2006, Zhou et al. 2011). Although here we focused mainly on osteogenesis, the Col-GM scaffolds can also be fine tuned

for various other tissue engineering applications by incorporating the necessary cues, which can be of interest in future.

5.4. Conclusions

In this study we have shown that, mechanical properties of collagen hydrogels can be modulated by incorporation of GMs which can also act as growth factor depots. Further, thus formed Col-GM scaffolds can provide a unique biomechanical and biomolecular environment to drive the encapsulated ADSCs towards osteogenic lineage. Moreover, increase in hydrogel rigidity seem to have an enchancing effect on the osteogenic differentiation of ADSCs as Col-20-GMs induce more differentiation compared to either Col or GM scaffolds alone. Such enhanced osteogenic induction was further increased by the incorporation of bFGF into the scaffolds as evident from the ALP activity results. Interestingly, we also observed high levels of BMP2 gene expression by ADSCs upon bFGF presentation within Col-GM scaffolds. BMP2 is known to play crucial role in bone regeneration and inducing the ADSCs to express it by themselves might reduce the need to supply BMP2 exogenously. Overall, we believe that, this kind of an injectable, *in situ* gelling, composite stem cell delivery system loaded with appropriate growth factors has the potential to facilitate the much needed transition of the ADSCs therapy to a clinical setting.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

This chapter provides an overview of the key findings from the studies presented in this thesis. In addition, a few recommendations which can take forward this work will also be discussed.

Stem cell therapies are creating exciting news time and again especially in the recent past with the outcomes of some of the clinical or experimental trials which were underway. Very recently, cure of HIV in two 'Boston patients' using stem cell transplantation has excited the whole world (Hayden 2013b). However, in five months time the HIV was found to be re-appeared again in those patients (Hayden 2013a). Similarly there are many other cases of clinical trials which have shown promising preliminary results but were still not been able to make it to next level (Chien 2004). There are also many instances where stem cell based clinical trials are failing or being terminated due to severe side effects (Kang et al. 2004). The reasons for such failures need to be fully addressed for these therapies to go ahead and attain their true potential. The work presented in this thesis is an effort made towards addressing few such issues with a focus of using human ADSCs for stem cell based tissue regenerative applications.

One of the main reasons for failure of cell therapies in general was found to be due to lack of cell adhesion sites once they were injected into the body as cell suspensions. Also the ischemic environment at the site of injury made the survival of injected cells difficult due to shortage of nutrient supplies. We tried to address this problem by developing cell-microsphere (ADSC-GMs) constructs that can provide cell adhesion sites for ADSCs and further investigated the tissue regenerative properties of these constructs including their pro-angiogenic potential. Further, to make these constructs feasible for a less invasive means of delivery, we encapsulated the cell-microsphere constructs in an injectable, *in situ* gelling collagen hydrogel to form hydrogel-microsphere composite scaffolds (Col-GMs). We also studied the effect of various mechanical as well as biomolecular cues provided by such a composite scaffold on the behaviour of ADSC-GMs.

Major findings obtained in both the above mentioned studies are summarized in the following sections.

6.1. Cell-microsphere constructs for tissue regenerative applications

In this study, we have fabricated cell-microsphere constructs with strong cell-cell and cell-matrix interactions by combining ADSCs and GMs. We then investigated

the suitability of ADSC-GM constructs for tissue engineering applications by studying certain key properties which play crucial role in tissue regeneration, namely – stemness maintenance, multi-lineage differentiation and pro-angiogenic potential.

Maintenance of stemness properties is crucial for stem cells to keep their multi-lineage differentiation abilities intact. Loss of stemness might lead to spontaneous differentiation of stem cells and thus can significantly reduce the number of undifferentiated stem cells available for tissue regeneration. To test stemness properties of ADSC-GMs and ADSCs on 2D, we studied the gene expression of well known pluripotent marker genes Oct4, Sox2, Nanog and Rex1. We found that all these genes except Rex1 were consistently down-regulated in ADSCs cultured on tissue culture plates both on day 3 and day 7. On the contrary, ADSCs cultured on GMs have up-regulated the expression of all the genes that were studied on both time points. This shows that, ADSC-GMs provide a unique stem cell niche which helps in preserving the pluripotent gene expression of ADSCs.

We then studied the multi-lineage differentiation abilities of ADSC-GMs by differentiating them into adipogenic, osteogenic and hepatic lineages and characterized using histo-chemical or immuno-fluorescent staining methods along with qPCR. Our staining results show that, ADSCs have successfully been able to differentiate into all the three lineages both on 2D and GMs. Further, qPCR studies for gene expression of lineage specific marker genes of all three lineages have shown that, ADSC-GMs express significantly higher amounts of the marker genes compared to ADSCs cultured on 2D. Differentiation of ADSCs towards adipogenic and osteogenic lineages has already been well established in various 3D scaffolds. However, hepatic differentiation of ADSCs has been relatively less studied especially in 3D scaffolds. Thus, the results obtained in this study show that 3D ADSC-GMs can also be used for liver tissue engineering applications along with fat and bone regeneration.

Finally, we also intended to study the pro-angiogenic potential of ADSC-GMs as vascularisation is also a key part of tissue regeneration. We have co-cultured the HUVECs (cultured on matrigel) either with ADSC-GMs or with ADSCs on 2D and studied the difference in the tube formation ability of the HUVECs among the

two groups. We found that, ADSC-GMs were able to induce significantly longer HUVEC tubules with more number of branch points compared to ADSCs on 2D indicating the superior pro-angiogenic activity of ADSC-GMs.

Overall, this part of our work shows that ADSC-GMs can maintain the stemness properties and enhance the differentiation and angiogenic properties. Thus, compared to traditional cell suspensions of ADSCs that are cultured on 2D culture plates, employing the ADSC-GM constructs for tissue regeneration can accelerate the wound healing process and also aid in the vascularization of the injured tissue.

6.2. Osteogenic induction of ADSCs in a hydrogel-microsphere composite scaffold

To make the ADSC-GM constructs suitable for injectable delivery and to maintain their location at the wound site, we encapsulated these constructs in a collagen hydrogel to form Col-GM composite scaffolds. Further, by controlling the mechanical and biomolecular cues in such Col-GM scaffolds we also tried to drive the ADSCs towards osteogenic lineage. Hydrogel rigidity was the mechanical cue and bFGF controlled release was the biomolecular cue that were employed to enhance the osteogenic induction. We found that incorporating GMs can reinforce the mechanical strength of the collagen hydrogels. Hence, we also tried to vary the matrix rigidity by varying the amount of GMs incorporated into collagen hydrogels.

Increase in matrix rigidity was found to enhance the osteogenic differentiation of ADSCs. Col-20-GMs which was found to have higher storage modulus compared to Col-10-GMs and Col gels, has also shown higher osteogenic induction in the encapsulated ADSCs.

To further accentuate the differentiation, we have encapsulated bFGF into the Col-GM scaffolds. bFGF controlled release experiments have shown a sustained release profile from all the three scaffolds with Col-20-GMs releasing at a much slower rate compared to GMs and Col gel. In addition, Col-20-GMs loaded with bFGF have also shown higher osteogenic differentiation. With slower release rates, Col-20-GMs were able to maintain higher amounts of bFGF within the scaffold for a longer time which thus might have better chance to interact with the

corresponding receptors on cell membranes and induce higher levels of osteogenic signals.

We also observed that, controlled release of bFGF was able to induce high levels of BMP2 gene expression in the encapsulated ADSCs during initial stages of differentiation. BMP2 is a growth factor well known for its pro-osteogenic activity (Bhakta et al. 2012). Many previous studies also indicated the synergistic effect of bFGF and BMP2 on bone regeneration (Hanada et al. 1997, Su et al. 2013). By inducing ADSCs to express BMP2 through bFGF controlled release, our scaffold system seems to be similar to dual delivery of bFGF and BMP2 which can have a positive impact on bone regeneration.

Overall, this study shows that by appropriately controlling the mechanical and biomolecular cues in the Col-GM composite scaffolds the differentiation of ADSCs towards a particular lineage can be enhanced, as we have shown here for osteogenic lineage. Thus, we believe, with the advantages of injectable delivery and regulating ADSC behaviour, the Col-GM scaffolds can aid in the transition of ADSC therapies to clinical stage.

6.3. Recommendations for future work

6.3.1. Modulating Col-GM scaffolds for other tissue engineering applications

The study presented in chapter 5 of this thesis mainly focused on osteogenic induction of ADSCs in Col-GM scaffolds. However, these scaffolds can also be fine-tuned for other tissue engineering applications by incorporating the necessary cues for a particular lineage. Appropriate mechanical cues can be provided by varying the amount of incorporated GMs accordingly. In addition, biocompatible crosslinkers which support *in situ* cell encapsulation (Liang et al. 2011) can also be employed if further increase in the matrix rigidity is required. Similarly, appropriate growth factors which promote differentiation towards a particular lineage can also be loaded into GMs and released at desirable rates.

As a preliminary study, we attempted to differentiate ADSCs in Col-GM scaffolds towards adipogenic lineage and characterized the differentiation by studying the gene expression of well-known adipogenic marker gene, PPAR-γ

using qPCR. As shown in Figure 5.9, we observed that, higher levels of PPAR-γ was expressed in softer gels such as pure collagen hydrogels or Col-10-GMs compared to the stiffer substrates like Col-20-GMs and GMs which is in agreement with the previous studies that have shown that softer gels can promote adipogenic induction in stem cells (Engler et al. 2006).

These studies involving osteogenic and adipogenic differentiation of ADSCs highlights the adaptability of the Col-GM scaffolds which further allows for designing the scaffolds, specific to different kinds of tissue engineering applications.

6.3.2. *In vivo* studies

All the tissue engineering approaches need to be tested in animal models to validate the results obtained under *in vitro* conditions and to confirm whether similar behaviour will be seen when injected *in vivo*. Many significant results were presented in this thesis which can contribute to the advancement of ADSC therapies and will be of interest to study if similar results can be obtained under *in vivo* conditions. For example, it will be interesting to study if the enhanced pro-angiogenic properties of ADSC-GMs (chapter 4) will also hold good under *in vivo* conditions. Further, it will also be of interest to study if the higher BMP2 expression observed in bFGF loaded Col-GM scaffolds (chapter 5) can have a positive effect on the bone regeneration *in vivo*.

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APPENDIX A

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Journal publications

1. **Anjaneyulu Kodali**, Thiam Chye Lim, David Tai Leong, Yen Wah Tong. Cell-microsphere constructs formed with human adipose derived stem cells and gelatin microspheres promotes stemness, differentiation and controlled pro-angiogenic potential. Macromolecular Bioscience. 14 (10), 1458-68; 2014.

2. **Anjaneyulu Kodali**, Thiam Chye Lim, David Tai Leong, Yen Wah Tong. Inducing osteogenesis in human adipose derived stem cells using mechanical and biomolecular cues in a gelatin microsphere – collagen hydrogel composite scaffold. (under communication).

Conference presentations

3. **Anjaneyulu Kodali**, Yen Wah Tong. *In Vitro* culture of human adipose derived stem cells and human hepatoma cells on gelatin microspheres. Tissue Engineering & Regenerative Medicine International Society (TERMIS) Asia Pacific Meeting. August 2011, Singapore.

4. Yen Wah Tong, **Anjaneyulu Kodali**. Gelatin microspheres as scaffolds for adipose derived stem cells and liver tissue engineering. American Institute of Chemical Engineers (AIChE) Annual Meeting, October 2011, Minneapolis, Minnesota, USA.

5. **Anjaneyulu Kodali**, Yen Wah Tong. Gelatin microspheres as scaffolds for culturing human adipose derived stem cells and human hepatoma cells. 14th Asia pacific Confederation of Chemical Engineering Congress, February 2012. Singapore.

6. Yen Wah Tong, **Anjaneyulu Kodali**. Microsphere - Collagen hydrogel with adipose derived stem cells for soft tissue engineering. American Institute of Chemical Engineers (AIChE) Annual Meeting, November 2013, San Francisco, California, USA.